A CLINICOMYCOLOGICAL STUDY OF ONYCHOMYCOSIS, ITS ANTIFUNGAL SUSCEPTIBILITY PATTERN AND RAPID DETECTION OF TRICHOPHYTON GENUS FROM NAIL SAMPLES BY PCR ANALYSIS OF 18S rRNA GENE INTERNAL TRANSCRIBED SPACER REGION

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CERTIFICATE

This is to certify that this dissertation entitled **"A CLINICOMYCOLOGICAL STUDY OF ONYCHOMYCOSIS, ITS ANTIFUNGAL SUSCEPTIBILITY PATTERN AND RAPID DETECTION OF TRICHOPHYTON GENUS FROM NAIL SAMPLES BY PCR ANALYSIS OF 18S rRNA GENE INTERNAL TRANSCRIBED SPACER REGION"** is the bonafide work done by **Dr.D.Dheepa** in the **Department of Microbiology, Govt. Stanley Medical College & Hospital, Chennai** in partial fulfillment of the regulation for **M.D(Branch – IV) Microbiology** examination of **The Tamil Nadu Dr.M.G.R. Medical University,** Chennai to be held in April 2012.

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DECLARATION

I, **Dr.D.Dheepa,** solemnly declare that this dissertation

"A CLINICOMYCOLOGICAL STUDY OF ONYCHOMYCOSIS, ITS ANTIFUNGAL SUSCEPTIBILITY PATTERN AND RAPID DETECTION OF TRICHOPHYTON GENUS FROM NAIL SAMPLES BY PCR ANALYSIS OF 18S rRNA GENE INTERNAL TRANSCRIBED SPACER REGION" is the bonafide work done by me at the Department of Microbiology, Govt. Stanley Medical College & Hospital, Chennai under the guidance and supervision of Dr.R.Selvi M.D, Professor and Head I/C, Department of Microbiology, Govt. Stanley Medical College, Chennai-600001.

This dissertation is submitted to The Tamil Nadu Dr.M.G.R Medical University, Chennai in partial fulfillment of the University regulations for the award of the degree M.D (Branch – IV) Microbiology examinations to be held in April 2012.

Dr. D. DHEEPA Place: Chennai

Date:

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CONTENTS

INTRODUCTION

Fungi are ubiquitous, capable of colonizing almost any environment.[124] The cutaneous infections in man include a wide variety of diseases in which the integument and its appendages – the hair and nail are involved.[123] The word onychomycosis in general describes fungal infection of the nails with the causative agent being dermatophytes, nondermatophytic moulds and yeasts.[91]

Onychomycosis is a common condition affecting 5.5% of the population worldwide and represents 20-40% of all onychopathies and about 30% of cutaneous mycotic infection .[40,7] Onychomycosis is a common nail ailment associated with significant physical and psychological morbidity. Increased prevalence in recent years is attributed to enhanced longevity, increase in comorbid conditions such as diabetes, avid sports participation and emergence of HIV.[11]
The prevalence rate of onychomycosis is determined by age, predisposing factors, social class, occupation, climate, living environment and frequency of travel.[92] In spite of improved personal hygiene and living environment, onychomycosis continues to persist and spread. Onychomycosis is not self-healing and may be a reservoir of more widespread fungal lesions of the skin. Although not life

threatening, this may have significant clinical consequences such as secondary bacterial infection, chronicity, therapeutic difficulties and disfigurement. The symptomatic disease can be a source of embarrassment and potential cause of morbidity in immunocompromised individuals.[136]

Dermatophytes, mainly *Trichophyton rubrum* and *Trichophyton mentagrophyte* var. *interdigitale* are responsible for nearly 90% of toenail onychomycosis and at least 50% of fingernail disease.[1,52]. However, yeasts (especially *Candida albicans*) and nondermatophyte moulds may also be implicated in previously traumatised nails.[100] Members of the genera *Scopulariopsis, Fusarium, Aspergillus, Geotrichum, Acremonium, Trichosporon, Alternaria, Sytalidium and Chaetomium* are increasingly being identified as primary pathogens of onychomycosis.[33]

Tinea unguium, onychomycosis due to dermatophytes is clinically undistinguishable from onychomycoses caused by nondermatophytes. Moreover, certain other conditions, such as psoriasis, lichen planus, onychogryphosis, and nail trauma can mimic onychomycosis. Hence laboratory investigations are needed to differentiate between fungal infection and the above mentioned skin diseases. An accurate diagnosis, with relevant laboratory

investigations is essential before staring treatment of onychomycosis.[69]. This is of paramount importance because the clinical outcome of antifungal agents varies as to whether the aetiologic pathogen is a dermatophyte, yeast or a mould.[122,93]

In contrast to other body sites onychopathies are difficult to eradicate with drug treatment and need prolonged treatment course. This is the consequence of factors intrinsic to the nail – the hard, protective nail plate, sequestration of pathogens between the nail bed and plate, and slow growth of the nail –as well as of the relatively poor efficacy of the early pharmacologic agents.[28] Recent developments in the application of nucleic acid amplification technology have proved to enhance the quality and rapidity of detection of the causative fungi.[16]

As the geographical distribution of fungi changes from time to time and since rapid and accurate diagnosis of onychomycosis is needed for the fact that therapy is directed by the type of organism,[121] this study, therefore, seeks to improve knowledge of the epidemiology, etiologic agent, clinico-mycological features, its antifungal susceptibility pattern and rapid identification of trichophyton from nail samples.

AIMS AND OBJECTIVES

1. To study the prevalence of onychomycosis and its aetiological agents.
2. To find out the commonest agent responsible for onychomycosis.
3. To study the correlation of fungal isolates and the clinical manifestations.
4. To detect *Trichophyton* genus directly from nail samples by PCR.
5. To compare the results of conventional methods and PCR.
6. To determine the antifungal susceptibility of the isolates by microbroth dilution.
7. To determine the antifungal susceptibility of filamentous fungi by E test method.
8. To compare the results of microbroth dilution and E test method for filamentous fungi.

REVIEW OF LITERATURE

HISTORY

Historically Agostino Bassi was the first to elucidate the microbial nature of decaying disease of silk worms. Robert Remak first observed hyphae in the scalp scrapings. Professor Johan Schonlein described the fungal etiology of favus in 1839 and Robert Remak described the etiological agent .[139] David Gruby was the real founder of medical mycology. He described the clinical entity favus and he described that fungi could be cultured and transmitted to humans.

Domenico Majocchi (1849-1929) first described variants of tinea. In 1892, Raymound Sabourad, a French dermatologist established the plurality of ring worm and described the mycological aspect.[88]

The nail unit is an indispensable part of the human body serving many useful functions such as protecting the digit, increasing the tactile sensitivity and picking small objects. The nail unit is also a universally considered best cosmetic attribute.[130] Any condition affecting the nail not only affects the person's ability to perform his daily activities but also can potentially diminish his quality of life.[132]

ANATOMY OF THE NAIL

The nail consists of four components – matrix, proximal nail fold, nail bed and hyponychium.[150]The nail plate is the largest structure of the nail unit and grows by sliding forward over the nail bed, where upon the distal end becomes the free edge.[42]The nail bed is an epidermal structure present under the nail plate. These cells keratinize and are slowly shed from the edge of the nail. Norton has demonstrated a distal motion to the epidermal cells of the nail bed.[113] Fingernails grow at a rate of 2 to 3 mm per month, and toenails grow at a rate of 1 mm per month. Hence, it takes about 6 months to replace a fingernail completely and around 12 – 18 months to replace a toenail.[133] This rate of growth is often decreased in the presence of peripheral vascular disease.[133]

DEFINITION OF ONYCHOMYCOSIS

The term "*Onychomycosis*" is a greek word *onyx* means nail and *mykes* means fungus .It was traditionally used to denote nondermatophytic infection of the nail but is now used as a general term to refer to any fungal infection of the nail[146](tinea unguium specifically describes a dermatophytic infection of the nail plate). Onychomycosis is thus a broad terminology which encompasses fungal infections of the nail caused by dermatophytes,

nondermatophytes and yeasts.Onychomycosis can have significant undesirable effects on the patients' emotional, social, and occupational functioning.. The disease puts the patients into great financial burden as the treatment is expensive and long duration of treatment is required for effective cure.[132] This in turn, can cause patients to feel discouraged or even to stop treatment by themselves, leading on to permanent disfigurement and discomfort. Onychomycosis in immunocompromised patients, such as those infected with human immunodeficiency virus (HIV), can pose a more serious health problem.[132] The disease not only becomes more resistant to treatment, but also poses a risk of higher rate of transmission of the disease to another person.[132]

Course of the disease and potential sequelea in high risk patients

Normal nail → exposure to fungus → colour changes →
thickened nail → ulceration → Necrosis and amputation

CLASSIFICATION (MODIFIED ZAAIAS CLASSIFICATION)

Onychomycosis can be classified according to the pattern of fungal invasion of the nail[150]

1. Distal and lateral subungual onychomycosis

In this type of onychomycosis, the nail involvement occurs at the distal and adjoining lateral borders of the nail. This occurs after the invasion of the horny layer of the hyponychium and nail bed. This type of onychomycosis was first described by the younger of the Mahon brothers.[98] . Distal and lateral subungual onychomycosis (DLSO) is usually caused by the dermatophyte *T.rubrum*,[54,137] although *T. mentagrophytes*, *T. tonsurans*, and *E. floccosum* also are known to be important causative agents.[137]

2. Proximal subungual onychomycosis

This is a very rare subtype with characteristic clinical appearance consisting of white areas extending distally from the proximal nail fold but more commonly involving the area of lunula. .[49]. *T.rubrum* is the principal causative agent of proximal subungual onychomycosis (PSO). Although PSO is the one of the most infrequently occurring forms of onychomycosis in the general population, it is very common in patients with HIV – AIDS and is considered an early clinical marker of HIV infection.[1,2] Infection may also occasionally arise secondary to trauma.

3. Superficial white onychomycosis

This condition was initially named *Leukonychia trichophytica* by Jessner in 1922 and *Leukonychia mycotica* by Rost in 1926.[87] Superficial white onychomycosis is characterised clinically by the appearance of well demarcated islands of opaque, white patches on the surface of the nail plate..[33] In addition, Zaias et al have implicated several nondermatophyte molds, including *Aspergillus terreus*, *Acremonium roseogrisum*, and *Fusarium oxysporum in causation of onychomycosis*.[150]

4. Candidial onychomycosis

Isolated involvement of the nail plate by *Candida albicans* is rare. Candidial onychomycosis usually occurs secondary to chronic paronychia. *Candida* nail infections also occur in patients with chronic mucocutaneous candidiasis.[7] These forms occur more commonly in women than in men[7] and often involve the middle finger, which usually comes in contact with *Candida* organisms that reside in the intestine or vagina.[150]The predisposing factors for this particular form of onychomycosis include frequent handling of water which results in damage and destruction of the cuticle. This denuded area forms the portal of entry for the fungus.

5. Total dystrophic onychomycosis

Total dystrophic onychomycosis is considered to be the endstage of nail disease, although some clinicians consider it a distinct subtype. It may be the end result of any of the four main patterns of onychomycosis. The entire nail unit becomes thick and dystrophic.[150]

6. Endonyx onychomycosis

It includes invasion of both the superficial and deeper nail plate. Lamellar splitting of the nail plate may be present. Pathogens include *T.soudanense* and *T.violaceum* which are associated with endothrix type of scalp infection.

EPIDEMIOLOGY AND PREDISPOSING FACTORS

Reports concerning the prevalence of onychomycosis are conflicting with estimates ranging from 2-3% to 13% in the western population. [5,55] Unlike in western countries where it is the frequent cause of nail disorders, in Southeast Asia the prevalence of onychomycosis is relatively low. This was partially confirmed by a large scale-survey in Asia in the late 1990s in which the prevalence of onychomycosis was lower in tropical countries (3.8%) than in subtropical countries and the countries in the temperate zone (18%).[24]

The prevalence rate of onychomycosis is determined by age, predisposing factor, social class, occupation, climate, living environment and frequency of travel.[147] The prevalence is higher (25%) in patients with human immunodeficiency virus infection (HIV).[67] Several studies have shown that prevalence of onychomycosis increases with age, reasons for which may include poor peripheral circulation, diabetes, repeated nail trauma, longer exposure to pathogenic fungi, sub optimal immune function, inactivity or the inability to cut the toe nails or maintain good foot care. [7] There is a considerably lower incidence of onychomycosis among children. The reasons for this 30-fold decrease in the prevalence of onychomycosis in children relative to adults may include reduced exposure to fungus because less time is spent in environments containing pathogens; faster nail growth, smaller nail surface for invasion and lower prevalence of tinea pedis. However, the worldwide prevalence of onychomycosis is increasing. In one study that evaluated the prevalence and risk factors of onychomycosis in individuals representing different strata of population in New Delhi the prevalence of onychomycosis was confirmed in 45% of the analysed patients. [92] . More recent evidence suggests that the

pathogens involved in onychomycosis may be more diverse than previously believed[42]

AETIOLOGICAL AGENTS

There are three groups of fungi associated with onychomycosis: yeasts.[92] The dermatophytes, non-dermatophytic moulds and fungi in each group have different growth requirements and this could explain their clinical behaviour of causing onychomycosis in the different habitats offered by nails in different individuals, and even different parts of the same nail.

Dermatophytes

The dermatophytes are the most common primary invaders of the nail. The onychomycosis caused by dermatophytes are referred to as tinea unguim. Their ability to digest and utilize keratin for their metabolic requirements forms the basis of their unique pathogenicity. In most cases, tinea unguim occurs secondarily from infection of the skin at nearby or distant sites. Frequent scratching could be a common predisposing factor resulting in onychomycosis..[134] The dermatophytic fungi commonly associated with onychomycosis include *T.rubrum, T.mentagrophytes, T.violaceum, T.tonsurans, E.floccosum*, etc. *T.mentagrophytes* usually infects the toe nails,

spreads very slowly and only rarely infects other body areas. *T.rubrum* in contrast spreads readily to involve both finger and toe nails and are considered to be more adapted and more virulent than *T.mentagrophytes*. Toe nails however can be attacked freely by both the species. *T.mentagrophytes* can also cause *leukonychia trichophytica*,[87] a type of surface infection of the nail plate. Such lesions are more typical of infection caused by moulds. The predominant species isolated include *T.rubrum*(31%), *T.violaceum*(5%), *T.mentagrophytes* (4%), *T.tonsurans*(2%) and *E.floccosum*(1%)[96,22] *T.rubrum* was found to be the commonest species followed by *T.mentagrophytes, Microsporum spp and E.floccosum*..[125]

Fungus	**Characters**	**Macroscopy**	**Microscopy**
T.rubrum	Anthropophilic Most common dermatophyte	Obverse; white downy to fluffy Reverse: yellow to blood red	Hyaline septate hyphae; sparse macroconidia, pencil or cigar shaped
T.mentagrophytes	Anthropophilic and zoophilic	Obverse;flat white granular Reverse; reddish brown	Microconidia in grape like clusters Macroconidia clavate to cigar shaped spiral hyphae
T.tonsurans	Anthropophilic	White to yellow Reverse-reddish brown	Hyphae with terminal swelling. macroconidia rare Microconidia in sleeve arrangement
T.verrucosum	Zoophilic	Knobby centre and folded colony	Chains of intercalary chlamydoconidia

		Reverse yellowish brown	
T.schonleinii	Anthropophilic	Heaped colony often submerged in the agar	Favic chandelier and antler hyphae
Microsporum audounii	Anthropophilic	Fluffy white and reddish brown reverse	Intercalary and terminal chlamydospore

Nondermatophytes

In the last three decades, there have been many reports documenting the role of nondermatophytes in causing onychomycosis.[138] The agents commonly implicated in onychomycosis include membersof *Scopulariopsis* (particularly *S. brevicaulis*) and *Scytalidium*, which are both believed to digest keratin in vivo, as well as members of the genera *Alternaria*, *Aspergillus*, *Acremonium*, and *Fusarium*.[138] Other nondermatophytic fungi that can cause onychomycosis include *Onychocola canadensis*, *Pyrenochaetaunguis-hominis*, and *Botryodiplodia theobromae*.[138] The most common yeast implicated is *C. albicans*.[138]

Organism	**Macroscopy**	**Microscopy**
Acremonium	Smooth, waxy and velvety with violet pigment	Hyaline septate hyphae. Slender conidiophores are phialides. Clusters of elliptical phialoconidia seen
Aspergillus flavus	Yellow green flat colony	Long conidiophore. Globose vesicle, foot cell and chains of conidia on full surface of vesicle seen.
Aspergillus niger	- Surface is covered with jet and black conidia with granular nature.	Globose vesicle at the tip of conidiophore. Chains of black prickly conidia in compact columns
Fusarium	Fluffy white colony with	Septate hyaline hyphae. Phialides arise

	varying colours. Often pink.	directly from hyphae. Both microphialoconidia and macrophialoconidia are crescent shaped.
- *Paecilomyces*	Flat and floccose ivory white colonies with light brown reverse	Conidiophores that are irregularly branched and chains of conidia bending away from conidiophore seen.
Rhizopus	Cottony to wooly gray colonies with salt and pepper appearance	Broad irregular hyphae and well developed rhizoids seen. Branching sporangiospores with hemispherical columella and growth at 25°C and 37°C.
Scopulariopsis	Mousy brown velvety colonies	Brush like pencillus arrangement. Large echinulate, lemon shaped anneloconidia with flattened base.
Syncephalastrum	Cottony-wooly gray to black colonies	Branching sporongiospore with globose vesicle supporting supporting tubular sporangia filled with sporangiospores
Trichosporon beigeli	Smooth waxy folded cerebriform folded colonies	Hyphae and pseudohyphae seen. Yeast form at 25°C and 37°C. Rectangular arthroconidia and blastoconidia seen. Positive urease reaction
Cladosporium	Dark olive green to black colonies	Cladosporium type of conidiation and pigmented hyphae seen
Exophiala jeanselmei	Young black yeast colonies	Pigmented annelids and anneloconidia seen. Annelids that form on annelophore and end in anneloconidia
Candida albicans	Yeast form of colonies	Blastoconidia and pseudohyphae present. Germ tube formation may be observed
Geotrichum candidum	Yeast form of colonies	Arthroconidia germinating in a hockey stick arrangement; noblastoconidia or pseudohypha

Scopulariopsis brevicaulis, Aspergillus and Acremonium spp are now known to infect the nails.[66] None of these fungi are keratinolytic. So they must survive on the intercellular cement substance or must derive benefit from the denaturation of nail keratin by old age, trauma or pre-existing disease. Walsh and English have shown that onychomycosis is more common in patients with skin

diseases which can cause nail changes and elderly people with senile nail changes.[145] English and Atkinson reported that in elderly individuals, double the proportion of onychomycosis occurred due to moulds(25%) as compared to dermatophytes(12%). Unlike dermatophytes, the nondermatophytic moulds colonize the outer layer of the nail.. Nondermatophytic infections more commonly involve the toe nails like the dermatophytes. However unlike dermatophytes, there is no question of involvement of the adjacent skin. Trauma serves as one of the most common predisposing events which result in the fungus getting lodged in the nail. Unlike the dermatophytes, the nondermatophytic moulds are capable of sporing in vivo. The conidia which are the reproductive spores of the dermatophytes are suppressed in the parasitic phase. This suggests that moulds grow as true saprophytes in the nail and are unaffected by the presence of living tissues in close proximity.[99].

Yeast

The ecology of the yeasts differ from both the dermatophytes and moulds. Candida albicans and Candida parapsilosis are the two most common yeasts isolated from nails. These occur usually secondary to chronic paronychia. Yeasts are neither keratinolytic nor

can actively colonize healthy nails. The diseased nail bed and nail fold not only form a focus of infection but also damage the nail as a result of which the fungus later spreads to involve the nail plate.

DIAGNOSIS OF ONYCHOMYCOSIS

The clinical presentation of dystrophic nails should alert the clinician to the possibility of onychomycosis; however, because fungal infections are responsible for only about half of all nail dystrophies,[52] the use of appropriate diagnostic methods including microscopy and culture is important to ensure correct diagnosis and treatment..[33] The difficulty in evaluating the role of nondermatophyte fungi cultured in the causation of onychomycosis arises because the same fungi that are common laboratory contaminants are also occasionally found to be pathogens.[137] All dermatophytes should be considered as pathogens. All the other isolated organisms must be considered as laboratory contaminants unless microscopy indicates that they have the atypical frond-like hyphae associated with nondermatophyte molds or if the same organism is repeatedly isolated. To increase the predictive power of a diagnosis of nondermatophytic nail infection, Summerbell suggested that non-filamentous nondermatophytes identified in nail tissue be categorized as one of the following: contaminant; normal

mammalian surface commensal organism; transient saprobiocolonizer; persistent secondary colonizer; successional invader; or primary invader .[137] By use of such an analysis, the clinician can identify for treatment only the nondermatophytic infections that are truly invasive.

Collecting the Nail Specimen

The first step of proper nail sample collection process is thorough cleansing of the nail area with alcohol to remove contaminants .[52]

Distal subungualonychomycosis

The dermatophytes causing DSO invade the nail bed rather than the nail plate. Hence the specimen must be obtained from the nail bed, with a small curette or a no. 15 scalpel blade.[52] where the concentration of viable fungi is greatest.[52]

Proximal subungualonychomycosis

A sharp curette must then be used to remove material from the infected proximal nail bed as close to the lunula as possible.[52]

White superficial onychomycosis

Since the infection involves the surface of the nail plate, a no. 15 scalpel blade or sharp curette can be used to scrape the white area and collect the infected debris.

Candidal onychomycosis

Material is required to be taken from the proximal and lateral nail edges. If *Candida* onycholysis is suspected, the nail plate should be lifted and the nail bed gently scraped..[53]

Specimen Analysis

Both direct microscopy and culture of sampled material are necessary to definitively identify the etiological agent.[52] The specimen should be divided into two portions – one for direct microscopy and the other for culture.

DIRECT MICROSCOPY; There are many limitations of direct microscopy when used for diagnosis of onychomycosis. The test serves only as a screening test for the presence or absence of fungal elements but cannot differentiate among the pathogens. Direct microscopy is often time-consuming, because nail debris may be thick and coarse and hyphae are usually only sparsely present.[52] There is also a fair chance of false-negative results, which occur at a rate of approximately 5 to 15%.[26,63] When examining a KOH mount, it is important to observe the hyphae closely and determine whether they are typical of dermatophyte fungi or have features of nondermatophyte moulds or yeasts. . In onychomycosis, direct microscopy is the most

efficient screening technique.[26, 63] The specimen can be mounted in a solution of 20 to 40% KOH or NaOH mixed with 5% glycerol, and heated to emulsify the lipids (1 hr at 51°C to 54°C), and examined . An alternative formulation consists of 20% KOH and 36% dimethyl sulfoxide.[146] The specimen may be counterstained with chitin-specific Chlorazol black E to accentuate hyphae that are present..[52] Parker blue-black ink also can be added to the KOH preparation to improve visualization.

CULTURE ; Culture is the only method by which the causative microorganism can be accurately identified. One should exercise caution while analysing culture results, because nails are nonsterile and other fungal and bacterial contaminants may obscure the original pathogen.[146] Specimens should be plated on two different media: a primary medium that is selective for dermatophytes alone; and a secondary medium that allows growth of contaminating nondermatophytic moulds and bacteria. Cycloheximide inhibits the growth of nondermatophytes and is incorporated into media such as dermatophyte test medium or Sabouraud peptone-glucose agar (Emmons' modification) with cycloheximide.[146] Cycloheximide-free media that are commonly used include Sabouraud's glucose agar, Littman's oxgall medium, and inhibitory mold agar (Sabouraud's

glucose agar with the addition of antibiotics).[52] If growth occurs on both types of media, the infective agent is probably a dermatophyte, whereas if growth occurs only on the cycloheximide-free medium, it indicates that the infective agent may be a nondermatophytic mould. However, growth of a nondermatophyte from a specimen that has tested positive for fungi on direct microscopy does not prove conclusively that the infective agent is a nondermatophyte.[63] The definitive identification of nondermatophytic invasion in nails may also require the isolation of the agent from successive specimens from the infected region.[63]

Treatment

Fungal infections are generally chronic and require long duration of treatment. The management of onychomycosis includes topical, systemic medication and surgical modalities.. Hence the earliest remedies were topical agents which were in wide use for treating dermatophytic skin infections including imidazoles (ketoconazole, econazole, and oxiconazole), allylamines(naftifine and terbinafine hydrochloride), and the pyridine ciclopiroxolamine.[46] ,flucanozole for candida and azoles like voriconazole and itraconazole and amphotericin B for treating nondermatophytic fungi.

ANTIFUNGAL SUSCEPTIBILITY TESTING

The incidence of fungal infection is on the increase since 1980. This should be considered as part and parcel of the diagnosis and treatment strategy. Susceptibility testing for antifungal drugs was virtually unknown in the 1980s but is now the focus of interest for research all over the world. Studies have shown extensive interlaboratory variation in the absolute MIC of antifungal drugs in the absence of standardized testing methods.[51] Antifungal susceptibility testing in infections such as onychomycosis becomes more clinically relevant when a choice of effective antifungal drugs exist. Onychomycosis responds well to systemic antifungal agents .The last 15 years have witnessed marked increases in the frequency of severe fungal infections as well as in the number of available antifungal agents. [2,18,20] The number of invasive infections caused by the filamentous fungi is lower than those caused by the yeasts. However, the emergence of new and less susceptible mould pathogens warrants the evaluation of their in vitro antifungal susceptibilities to both established and investigational antifungal agents. [1,3,17,18] Work on development of meaningful and standardized procedures for testing of yeasts has led to the publication of the document M27-T (tentative

standard) by the National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee on Antifungal Susceptibility Testing.[61] The endeavors of the NCCLS Subcommittee have recently been directed towards developing standard guidelines for the antifungal susceptibility testing of filamentous fungi.[107] Initial work demonstrated that reliable conidial suspensions could be prepared by a spectrophotometric procedure.[61] Based on these results, the NCCLS Subcommittee adapted other testing variables of the M27-T method to the filamentous fungi.[107]

RECENT MOLECULAR METHODS

Dermatophytes are among the few fungi causing communicable disease. Previously most dermatophyte strains had relatively restricted geographic distribution. But nowadays dermatophytosis seems to be one of the commonest diseases worldwide. It cannot be diagnosed only based on clinical criteria.[20] It is difficult to detect in immunocompromised patients due to atypical presentation.[114] Further dermatophytes from patients on antifungal treatment do not show characteristic culture and morphology which compromises the result of culture isolation.[95] The changing profile of human dermatophytosis needs the development of improved and rapid

methods for identification of dermatophytes.[134] Thus newer fungal identification methods are the need of the hour as identification is required not only for diagnosis but also for therapeutic strategies. A simple, rapid and sensitive method for the diagnosis of dermatophyte infection definitely will be a major improvement. Recently molecular biology based techniques such as PCR, real time[10] and multiplex PCR[134]have been adopted for detection of dermatophytes from clinical samples. They have good potential to directly detect dermatophytes from clinical samples. In a study, Nagao et al[106] detected Trichophyton rubrum by nested PCR by targeting internal transcribed spacer (ITS). Yan et al[148] evaluated arbitrary primed PCR for detection of dermatophytes. Bergman et al[21] performed a PCR reverse line blot assay and Garg et al[85] detected dermatophytes targeting chitin synthase.

PREVENTION

Even with apparently optimal diagnosis and treatment, one in five onychomycosis patients are not cured by current therapies. The reasons for the 20% failure rate are inaccurate diagnosis, misidentification of the pathogen, presence of a second disorder, characteristics of the nails, presence of a high fungal inoculum and/or

drug-resistant microorganisms, compromised immune system of the host, diabetes mellitus or peripheral vascular disease.

Following are some suggested measures:

Avoid going barefoot in public places, Keep feet cool and dry., Apply topical antifungal medication regularly to the feet and toe nails.,Discard Old shoes and "rest" shoes periodically to decrease their exposure to fungi.,Apply an antifungal powder/spray to the inside of shoes once a week or more.Comply with the treatment protocol.[122]

MATERIALS AND METHODS

STUDY DESIGN - Prospective cohort study

The present study was carried out in Department of Microbiology in Govt. Stanley Medical College Hospital, Chennai.

STUDY PERIOD : OCTOBER 2010 -AUGUST 2011

SAMPLE SPECIFICATION:

Nail scrapings were collected from one hundred fifty patients of clinically diagnosed cases of onychomycosis in mycology section of the Dermatology outpatient department at Govt. Stanley Medical College and Hospital, Chennai.

INCLUSION CRITERIA:

All patients with clinically diagnosed case of onychomycosis irrespective of age and sex.

EXCLUSION CRITERIA:

Patients with nail changes due to psoriasis, paronychia and lichen planus and those who are under antifungals.

A detailed history of occupation, comorbid condition, and medical history were taken from all patients. Consent was obtained from all patients.

ETHICAL COMMITTEE CLEARANCE:

This study has been approved by ethical committee.

SAMPLE COLLECTION:

Collecting the Nail Specimen

First a thorough cleansing of the nail area should be done using alcohol to remove contaminants. Because the site and localization of infection differ in the different types of onychomycosis, different approaches, depending on the presumptive clinical diagnosis, are necessary to obtain optimal specimens.

Distal subungual onychomycosis

The nail was clipped short with nail clippers, and sufficient material was taken from the nail bed as proximal as possible to the cuticle and from underside of the nail plate with a no. 15 scalpel blade

Proximal subungualonychomycosis.

The healthy nail plate was gently pared away with a no. 15 scalpel blade and material from the infected proximal nail bed as close to the lunula was removed using a curette.

White superficial onychomycosis

With the use of a no. 15 scalpel blade, white area of the nail was scraped and collected.

***Candidal* onychomycosis**

Material was collected from the proximal and lateral nail edges.

Total nail dystrophy

Material was collected from the dystrophied site by using no.15 scalpel blade

TRANSPORT OF SAMPLE

The samples were collected in black sterile paper sachets such that the scrapings are easily seen.

SAMPLE PROCESSING

The sample was divided into three portions for direct microscopy, culture and for PCR.

TUBE KOH

The scrapings were placed in a tube of 20% KOH with 40% DMSO and after three hours a drop of the solution from the tube was placed on a slide and a cover slip was placed on it and seen under low power and high power. A drop of LPCB was added for better visualization. Fungal hyphae, spores, pseudohyphae and yeast cells could be clearly seen.

CULTURE

The nail sample collected was crushed into twenty inoculums and ten of them were inoculated in Emmonds modified Sabourads dextrose agar containing chloramphenicol (50 mgs/l) and cycloheximide (500mg/l) and the remaining ten inoculums in SDA with chloramphenicol to detect the growth of other nondermatophytes. All the slopes were incubated at 25ºC and examined twice a week for evidence of fungal growth. Slopes not showing growth for 4 weeks were discarded. If the isolate is a dermatophyte then they were inoculated on to a potato dextrose agar for better conidiation.Slide culture ,cellophane tape method and tease mount was done to study the microscopic characters . Speciation was done later based on their characters. Diagnosis of nondermatophytic onychomycosis was made according to English and Walsh criteria[145,57,58]

Nail abnormalities consistent with the diagnosis

Positive KOH preparation in the presence of hyphae in nail keratin.

Failure to isolate the dermatophyte in culture.

Growth of more than five colonies of the same mould in at least two consecutive nail samples.

Specimens that were positive by direct microscopy but failed to grow were cultured repeatedly. If the mould was isolated at first

culture, its etiologic significance for nondermatophytic onychomycosis was confirmed after third positive culture was examined and non dermatophytes were identified on the basis of their macroscopic and microscopic features. If the isolate is an yeast then gram stain, germ tube test, chrome agar and cornmeal agar was used for differentiation.

Slide culture

It is used to study the undisturbed morphological details of fungi. A sterile microscopic slide was placed on a bent glass rod at the bottom of a petridish. A piece of one cm^2 block of potato dextrose agar was put up on the slide. The fungal strain to be isolated was inoculated on the four sides of the block. The inoculated block was covered with a cover slip and distilled water was placed in the bottom of the petridish such that agar doesn't dry out. When growth appears a drop of LPCB was placed on the slide and covered using a coverslip. A drop of LPCB can also be applied on the slide after removing the agar.

Cellophane tape method

This is a instant slide culture method. A piece of tape was gently laid over portion of the fungal growth and slowly lifted to remove an area and was then placed on a drop of LPCB and examined under low and high power.

UREASE TEST

The test was done in Christensen urease agar to differentiate *T.rubrum* from *T.mentagrophytes*; *T.mentagrophytes* hydrolyse urea whereas *T.rubum* doesn't. Among nondermatophytes *Trichosporon* hydrolyse urea where *Geotrichum* doesn't.

GRAM STAIN

Gram stain was performed on all the yeast and yeast like isolates.

GERM TUBE TEST (REYNOLDS BRAUDE)

A small inoculum of yeast cell was suspended in 0.5 ml of human serum and incubated at 37ºC for 2 hours and then a drop of suspension was placed in a slide, a coverslip was placed over it and examined for the presence of germtube. Germ tube will be an appendage of one half of width and length thrice of the yeast cell. *C.albicans* and *C.dublininensis* will produce germ tube.

CORNMEAL AGAR

An isolated colony from SDA was inoculated on a plate of cornmeal agar containing 1% Tween 80 by making three parallel streaks about ½ inch apart and at an angle of 45º to the culture medium. A sterile coverslip was placed on the streak line. The plate was incubated at 30ºC for 48 hours. The plate was examined at the edge of the streak and the coverslip for the presence of blastoconidia, arthroconidia, pseudohyphae and chlamydoconidia.

Organism	**Blastoconidia**	**Hyphae/pseudohyphae**
C.albicans	Spherical cluster at regular intervals on pseudohyphae	Chlamydoconidia
C.glabrata	Small spherical and tightly compacted	None produced
C.parapsilosis	Present but not characteristic	Giant hyphae present
C.kefyr	Elongated lie parallel to pseudohyphae	Pseudohyphae present
C.tropicalis	Produced randomly along hyphae	Pseudohyphae present

CHROME AGAR MEDIA

Isolated colony was inoculated on chrome agar medium and incubated at 30º for 72 hrs and observed for the colour of colonies,

Candida	*Colour*
C.albicans	Light green
C.parapsilosis	Cream
C.tropicalis	Blue with pink halo
C.krusei	Pink
C.glabrata	Purple

C.dublinensis	Dark green

ANTIFUNGAL SUSCEPTIBILITY TEST:

Microbroth dilution for dermatophytes (M 38 A) and for filamentous fungi (M38P) and for yeast as per M27P document. The in vitro activity of triazole antifungal agents, voriconazole, itraconazole and Amphotericin B using the National Committee for Clinical Laboratory Standards (NCCLS) M38-P microdilution method [109,110,11] for filamentous fungi. Fluconazole, amphotericin - B, terbinafine, griseofulvin, and ketaconazole were tested against dermaophytes using M -38 A document. All the drugs are obtained as reagent-grade powders from their respective manufacturers. Stock solutions were prepared in distilled water (flucanozole) and in dimethyl sulfoxide (itraconazole, voriconazole, and amphotericin - B). (SIGMA)

WEIGHING ANTIFUNGAL POWDERS:

$$\text{Weight(mg)} = \frac{\text{volume(ml)} \times \text{concentration (µg/ml)}}{\text{Assay potency(µg/ml)}} \quad \text{or}$$

$$\text{Volume(ml)} = \frac{\text{Weight(mg)} \times \text{Assay potency(µg/ml)}}{\text{concentration (µg/ml)}}$$

MEDIUM

All drugs were diluted in RPMI 1640 medium (Sigma Chemical Co.) with 0.3 g of L-glutamine per liter and 0.165 M MOPS buffer (34.54 g/liter) and without sodium bicarbonate buffered to pH 7.0 dispensed into 96-well microdilution trays.

The recommendations stated in NCCLS document M38-P were followed for the dilution of each antifungal agent.[23]

ANTIFUNGAL STOCK

Flucanozole (64-0.0625µg), ketaconazole, itraconazole, voriconazole, amphotericin – B and terbinafine (32-0.0313µg). It should be prepared at a concentration of atleast ten times the highest concentration to be tested. 5 ml of stock to be prepared.

Water soluble drugs:

Twofold dilutions of a water soluble antifungal agent is used and prepared volumetrically in broth.

Water insoluble drugs:

For example to prepare a dilution test for a drug whose highest concentration is 16µg/ml weigh out 4.8 mg (assuming 100% potency) of antifungal powder and dissolve in 3.0ml of DMSO which will provide a stock of 1600µg/ml and further dilutions to be prepared as

per annexure. The solution in DMSO will be further diluted 1:50 in the test medium and a further twofold when inoculated.

DRUG DILUTION:

For example if a series with final concentration of 16µg/ml to 0.0313µg/ml is desired then a concentration series of 1600 to 3.13µg /ml is prepared in DMSO. To prepare 5ml of diluted antifungal agent, 4.9ml of RPMI 1640 is first pipetted into each of 10 sterile test tubes. Now using a single pipette 0.1ml of DMSO alone is added to one 4.9 ml lot of medium (control); then 0.1ml of the lowest concentration to 4.9 ml of medium and then continued in sequence up the concentration series. Since there is a 1:2 dilution of the drugs when combined with this inoculum the working antifungal are twofold more concentrated than final concentration re used. The final ranges of drug concentrations tested were 0.0313-32 µg.

INOCULUM PREPARATION:

Filamentous fungi:

All isolates were stored as spore suspensions in sterile distilled water at room temperature until they were used in the study. Before testing, each isolate was subcultured at least twice on potato dextrose agar (Remel,.) to ensure its viability and purity.

Susceptibility testing:

MICs were determined by the NCCLS M38-P broth microdilution methodology. Briefly, each isolate was grown on potato dextrose agar slants at 35°C for a period of 7 days. The fungal colonies were then covered with 1 ml of sterile 0.85% saline and gently scraped with a sterile pipette. The resulting suspensions were transferred to sterile tubes, and heavy particles were allowed to settle. The turbidity of the conidial spore suspensions was measured at 530 nm. The densities of the conidial suspensions were read and adjusted to an optical density (OD) that ranged from 0.09 to 0.11 (80 to 82% transmittance) for *Aspergillus* spp. and 0.15 to 0.17 (68 to 70% transmittance) for *Fusarium spp.*, *P.boydii*, and *R. arrhizus*[5,7] . Each well was inoculated with 100 µl of the 2x inoculum and 100µl of the drug suspension. The plates were incubated at 28ºC for 24 hr or less (*R. arrhizus*) to 5 days dematiceus fungi and for 24-48 hours for other filamentous fungi.

Incubation and MIC determination:

For the conventional procedure, the growth in each MIC well was compared with that of the growth control with the aid of a reading mirror. The microdilution wells were then given a numerical score as

follows: 4 - no reduction in growth; 3 - slight reduction in growth or approximately 75% of the growth control; 2 – prominent reduction in growth or approximately 50% of the growth control; 1 – slight growth or approximately 25% of the growth control; and 0 - optically clear or the absence of growth.

YEAST (M 27 P) document ;[107.108]

5 colonies of *Candida* is picked up and suspend in 5 ml of sterile 0.85% saline and adjust such that turbidity matches 0.5 Mc Farland standard. It produces a cell suspension of $1x10^{6}$ to $5x10^{6}$ CFU /ml. Remaining procedure of stock preparation and drug dilution are same as filamentous fungi.

E-test procedure

The E-test was performed by following the manufacturer's instructions. Each solidified medium was inoculated by dipping a nontoxic (latexfree) sterile swab into the respective undiluted stock inoculum suspension and evenly streaked it in three directions over the entire surface of a 120-mm petri plate containing 25 ml of medium; the swab was dipped into the inoculum suspension each time that the agar surface was streaked . The agar surface was allowed to dry for 15 min, and the strips were placed onto the inoculated agar.

The plates were incubated at 35°C, and the MICs were determined following incubation for 24 hrs to 4 days. The MICs determined by the E-test were the lowest drug concentrations at which the border of the elliptical inhibition intercepted the scale on the antifungal strip .The MICs were determined by the visual inspection of growth inhibition as described in the NCCLS M38-P document[22] and corresponded to complete growth inhibition.

DERMATOPHYTES[87] :

Preparation of inocula

The isolates were transferred from sterile saline (0.9%) to potato dextrose agar at 28°C for 7 days to produce conidia. The fungal colonies were covered with 5 ml of sterile saline (0.9%), and the suspensions were made by gently probing the surface with the tip of a Pasteur pipette. The mixture of conidia and hyphae fragments was filtered with a Whatman filter model 40 (pore size, 8 μm), which retains hyphal fragments and permits passage of only microconidia. The densities of these suspensions were adjusted with a spectrophotometer at a wavelength of 520 nm to a transmittance of 70 to 72%. The inoculum sizes ranged from 2×10^6 to 4×10^6 CFU/ml.

Test procedure

Flat-bottomed microdilution plates (96 wells) were set up in accordance with the NCCLS reference method.[16] Each microdilution well containing 100 µl of the twofold drug concentration was inoculated with 100 µl of the diluted inoculum suspension. For each test plate, two drug-free controls were included, one with the medium alone (sterile control) and the other with 100 µl of medium plus 100 µl of inoculum suspension (growth control). The microdilution plates were incubated at 28°C and 35°C and were read visually after 7 days of incubation.

Reading and interpretation of MICs

Endpoint determination readings were performed visually based on comparison of the growth in wells containing the drug with that of the growth control. For azole agents and for griseofulvin, the MIC was defined as the lowest concentration showing prominent growth inhibition (a drop in growth corresponding to approximately 80% of the growth control. For terbinafine, the MIC was defined as the lowest concentration showing 100% growth inhibition. MIC ranges of each drug were obtained to facilitate comparisons of the activities of tested drugs, as well as readings of the MIC at which 50% of the isolates

were inhibited (MIC50); similarly, MIC90 is the MIC at which 90% of the isolates were inhibited.

PCR PROTOCOL

Optimization of PCR

PCR on the nail samples included extraction of genomic DNA from nail samples followed by amplification using primers specific for 18S rRNA gene ITS 1 region of *Trichophyton* (200bp).

DNA EXTRACTION

All the reagents and the primers were obtained from Helini® biomolecules, Chennai. The water bath or heating block is preheated to 100ºC before beginning the procedure. Then 1 – 5mg of nail scrapings or loop full of culture is added into 1.5ml tube. Then 300µl of Ready Template **A** and 300µl of Ready Template B was added and for 30 seconds. Using micropestle the mixture was homogenized thoroughly. The samples are then incubated for 10 minutes in a 100ºC water bath or heating block and centrifuged at top speed (10000rpm – 15000rpm) for 5minutes at room temperature. 50µl of the supernatant is transferred carefully to fresh 1.5ml sterile tube and stored at- 20ºC.

AMPLIFICATION

10µl of Helini® RedTaq master mix is taken in a PCR tube. 1µl of internal control primer mix targeting human DNA at 500bp is added. 1µl of Trichophyton primer mix targeting internal transcribed spacer at 200bp is added. 1µl of DNA from the nail sample is then added. Negative control as the culture of nondermatophytes is included. ATCC T.rubrum 34265 was also included as controls . DNAase free milliq water is added such that the final concentration is 20µl. DNA from *Trichophyton* culture samples and was also included. All PCR steps were carried out in 20µl reaction volume in polypropylene tubes using a Perkin Elmer thermal cycler. The PCR profile consists of initial denaturation at 95°C for 3min, 35 cycles of denaturation at 95°C at 30seconds, annealing at 62°C for 30seconds, extension at 72°C at 30seconds, final extension at 72°C for 5minutes.

DETECTION OF AMPLIFIED PRODUCTS

The products were visualized by running the products in 2% agarose gel electrophoresis incorporated with 5µl of ethidium bromide at 100 volt for 20 minutes using a UV transilluminator (302nm). Helini® 100bp DNA ladder was used.Trichophyton culture yield band at 200bp . Nail samples with *Trichophyton* positives will show band at 500 bp (internal control –human genome)and and at 200bp,(ITS I) of *Trichophyton*.

ANALYTICAL SENSITIVITY AND SPECIFICITY

Analytical sensitivity and specificity were calculated by means of using ATCC *T.rubrum* 34265.the analytical sensitivity of primers was 50 copies /ml of ATCC strain. The primers were specific such that it can amplify all the Trichophyton isolates tested and not amplifying the other nondermatophyte fungal DNA.

RESULTS

150 nail samples were collected and processed. All the samples were subjected to KOH with DMSO and culture. Antifungal susceptibility testing was done by microbroth dilution method . PCR was done to detect *Trichophyton* from nail samples. Statistical analysis was done using SPSS version 15. P VALUE $\leq$ 0.05 was considered statistically significant. Results are as follows:

Table 1: GENDER RATIO

GENDER	**FREQUENCY**	**%**
MALE	93	62%
FEMALE	57	38%
TOTAL	**150**	

Of the total 150 patients, males (62%) were seen to predominate over females. (38%)

Table 2: AGE DISTRIBUTION

AGE	**MALE**	**FEMALE**	**TOTAL**
<15	7	1	8 (5.3%)
16 – 25	12	12	24 (16%)
26 – 45	34	28	62 (41.3%)
46 – 65	23	10	33 (22%)
>65	17	6	23 (14.7%)

Out of 150 patients, maximum cases were observed to be between 26 – 45 yrs (41.3%). The youngest patient was 6 yrs old and the oldest patient was 74 years old. The mean age was observed to be 40.24. Standard deviation was found to be 17.64.

Table 3: OCCUPATION

OCCUPATION	MALE	FEMALE	TOTAL	P VALUE
Farmers	31	8	39 (26%)	0.003
Office workers	18	6	24 (16%)	0.634
Housewife	-	21	21 (14%)	0.385
Students	10	5	15 (10%)	0.361
Labourers	31	12	43 (28.67%)	0.001
Miscellaneous	4	4	8 (5.3%)	1.124

Labourers topped the list of patients affected with onychomycosis (28.67%) followed by farmers (26%) among both males and females. Among females alone, housewives formed the majority of patients. P value was noted to be <0.001 for labourers and farmers which have a significant association with onychomycosis.

Table 4: PREDISPOSING FACTORS AND COMORBID CONDITIONS

CONDITION	NO OF PATIENTS	P value
Trauma	54(36%)	0.001
Diabetes	30(20%)	0.105
Immunosuppressive condition	8(5.33%)	0.119

54 patients (36%) gave history of trauma, while 30 (20%) had diabetes mellitus and 8 (5.33%) gave a history of immunosuppressive conditions. Trauma (P value ≤0.01) is found to be significantly associated with onychomycosis.

Table 5: TYPE OF ONYCHOMYCOSIS

Type	Finger nail	Toe nail	Both	Total
Distal lateral subungual onychomycosis	57	25	14	96(64%)
Proximal subungual onychomycosis	5	2	0	7(4.66%)
Superficial white onychomycosis	8	7	0	15(10%)
Total dystrophic onychomycosis	12	20	0	32(21.33%)
Endonyx type	0	0	0	0
Total	82(54.66%)	54(36%)	14(9.33%)	

Among 150 patients, 96 (64%) had DLSO (Distal lateral subungual onychomycosis). Among all patients, finger nails were seen to be predominantly involved 82 (54.66%). Toe nails were involved in 54 patients (36%) and both the nails in 14 (9.33%).

Table 6: TOTAL NO OF ISOLATES

DERMATOPHYTES	NON DERMATOPHYTES	CANDIDA	NO CULTURE	TOTAL
45 (58.44%)	27(35.06%)	5(6.49%)	73(48.67%)	77

Out of 150 cases, culture was positive in 77 (51.33%). Among the isolates recovered, dermatophytes were seen to predominate (58.44%). Nondermatophytes were cultured from 35.06% cases and candidal infection was seen in 6.49% of cases.

Table 7: TOTAL NUMBER OF DERMATOPHYTES ISOLATED WITH RESPECT TO TYPE OF ONYCHOMYCOSIS

Organism	**DLSO**	**PSO**	**SWO**	**TDO**	**Endonyx**	**Total (45)**	**%**
T.rubrum	14	0	0	4	0	18	40.
T.mentagrophytes	1	4	3	0	0	8	17.77
T.schonleinii	8	0	0	0	0	8	17.77
T.verrucosum	3	0	0	2	0	5	11.11
T.tonsurans	2	0	0	3	0	5	11.11
M.audounii	1	0	0	0	0	1	2.22

T.rubrum was the predominant dermatophyte isolated constituting 40% and 77.77% has been predominantly isolated from DLSO type of onychomycosis.

Table 8: EXTRA UNGUAL SITES INVOLVED

ORGANISM	**Trunk/Face /head**	**Groin**	**Feet**	**Hands**	**Finger nail**	**Toe nail**
T.rubrum	8	10	14	4	14	4
T.mentagrophytes	0	0	8	0	2	6
T.shonleinii	8	0	0	1	5	3
T.verrucosum	4	1	0	1	4	1
T.tonsurans	4	2	0	0	5	0

M.audounii	1	0	0	0	1	0

Groin and feet were seen to be the predominant extra ungual site involved in patients with onychomycosis due to *T.rubrum*. *T.mentagrophytes* was also seen to be associated with tinea pedis. *T.verrucosum, T.schonleini, T.tonsurans, M.audounii* were associated with tinea capitis.

<u>Table 9</u>: TOTAL NO OF NONDERMATOPHYTES AND YEAST ISOLATED WITH RESPECT TO TYPE OF ONYCHOMYCOSIS

ORGANISM	**DLSO**	**PSO**	**SWO**	**TDO**	**Endonyx**	**TOTAL (32)**	**%**
Fusarium	5	-	2	-	-	7	25.95%
Candida	3	2	-	-	-	5	7.41%
Acremonium	2	-	-	-	-	2	3.70%
Exophiala	-	-	-	1	-	1	7.40%
Cladosporium	2	-	-	-	-	2	3.70%
Sytalidium	1	-	-	-	-	1	7.40%
Trichosporon	-	2	-	-	-	2	7.40%
Geotrichum	1	-	1	-	-	2	7.40%
Syncephalastrum	-	-	-	2	-	2	7.40%
Scopulariopsis	-	2	-	-	-	2	7.40%
Aspergillusniger	2	-	-	-	-	2	7.40%
Paecilomyces	2	-	-	-	-	2	3.70%

Rhizopus	1	-	-	-	-	1	3.70%
Aspergillusflavus	1	-	-	-	-	1	

Among the nondermatophytes, *Fusarium* was the most common species isolated (25.95%). *Acremonium*, *Cladosporium*, *Trichosporon*, *Geotrichum*, *Syncephalastrum*, *Scopulariopsis*, *Aspergillus niger* and *Paecilomyces* were positive in 7.40% cases each respectively. *Exophiala*, *Scytalidium*, *Rhizopus* and *Aspergillus flavus* were positive in 3.70% cases each respectively..Non dermatophytic molds (74.07%) were seen to be predominantly associated with DLSO. isolated from DLSO (60%) and PSO (40%).

Table 10: NONDERMATOPHYTES AND THEIR EPIDEMIOLOGICAL PATTERN (total=27)

AGE		**SEX**		**OCCUPATION**		**COMORBID**		**NAIL INVOLVEMENT**		**TYPE**	
<50	2	Male	22	Farmers	18	Trauma	17	Toe	23	DLSO	17
>50	25	Female	5	Labourer	5	Diabetes	5	Finger	4	PSO	4
				Others	4	Immuno suppression	5			SWO	3
										TDO	3

Nondermatophytes were more common in elderly (92.59%) (p value =0.001) and in males (81.48%). (p value =0.03) With regard to occupation majority were farmers (66.66%) (p value =0.005) followed by labourers (18.51%) .The major predisposing factor was trauma (62.96%) (p value =.0.01). DLSO was found to be

the most common type of onychomycosis. (62.96%) (p value =0.013). It was predominantly isolated from toe nails (85.19%) (p value =0.01).

Table 11: EXTRA UNGUAL SITES INVOLVED

ORGANISM	Trunk	Groin	Feet	Hands	Finger nail	Toe nail	Total
Fusarium	-	-	-	-			7
Candida	5	3	2	-	4	1	5
Acremonium	-	-	-	-	-	2	2
Exophiala	-	-	-	-	-	1	1
Cladosporium	-	-	-	-	-	2	2
Sytalidium	-	-	-	-	-	1	1
Trichosporon	-	-	-	-	2	-	2
Geotrichum	-	-	-	-	2	-	2
Syncephalastrum	-	-	-	-	-	2	2
Scopulariopsis	-	-	-	-	-	2	2
Aspergillusniger	-	-	-	-	-	2	2
Paecilomyces	-	-	-	-	-	2	2
Rhizopus	-	-	-	-	-	1	1
Aspergillus flavus	-	-	-	-	-	1	1

Among 27 nondermatophytes, *Candida* was the species seen to commonly involve the extra ungual sites. All the Candida species (80%) were isolated predominantly from fingernail of housewives. EAmong the other nondermatophytes, (85.19%) had toe nail involvement. Finger nail involvement was seen in (14.81%) of isolates. *Trichosporon* and *Geotrichum* were isolated from finger nails.

Table 12: YEAST ISOLATES(n=5)

YEAST	NUMBER	PERCENTAGE
Candida albicans	3	60%
Candida tropicalis	1	20%
Candida parapsilosis	1	20%

Candida was isolated in 5 (6.49%) of cases. Among *Candida*, *C.albicans* (60%) was the predominant species isolated. Other species isolated include *C.tropicalis* (20%) and *C.parapsilosis* (20%).

Table 13: COMPARATIVE ANALYSIS OF KOH AND CULTURE FOR ALL CASES(TOTAL =150)

Method	No of positives	%
KOH(DMSO)	86	57.33
CULTURE	77	51.33

Overall KOH positivity for all cases was observed to be 57.33% and fungal culture positivity 51.33%

Table 14: POSITIVITY RATE OF CONVENTIONAL METHODS IN TRICHOPHYTON DETECTION (TOTAL =117)

METHOD	NO	%
KOH(DMSO)	53	45.30
CULTURE	44	37.60

Among the remaining 117 cases, (after excluding nondermatophyte culture positive cases, *Candida* and *Microsporum audounii*) KOH mount was observed to be positive in 53 cases (45.30%), culture in 44cases (37.60%)

Table 15: POSITIVITY RATE OF CONVENTIONAL METHOD TRICHOPHYTON DETECTION (TOTAL AND PCR FOR =117)

TEST	KOH MOUNT(DMSO)	CULTURE	PCR
NO OF CASES POSITIVE	53	44	66
POSITIVITY RATE (%)	45.30	37.60	56.41

Among the remaining 117 cases, (after excluding nondermatophyte culture positive cases, *Candida* and *Microsporum audounii*) KOH mount was observed to be positive in 53 cases (45.30%), culture in 44cases (37.60%) and PCR in 66 cases (56.41%).

Table 16: COMPARATIVE ANALYSIS OF KOH, CULTURE AND PCR IN TRICHOPHYTON DETECTION

No of isolates	KOH	Culture	PCR
44 culture isolates	Positive	Positive	Positive
7 nail samples	Positive	Negative	Positive
2 nail samples	Positive	Negative	Negative
13 nail samples	Negative	Negative	Positive
51 nail samples	Negative	Negative	Negative

PCR analysis was done for all 117 cases after excluding 27 nondermatophytes, 5 *Candida* and one *Microsporum* isolates. All the 44 cases which were positive by KOH and culture were also positive with PCR. Out of 9 isolates which were positive by KOH mount and negative by culture only 7 were shown to be positive with PCR and two of them were negative. Additionally, 13 cases which were negative by both KOH mount and culture were positive by PCR. The remaining 51 cases which were negative by both KOH mount and culture were also seen to be negative with PCR. PCR was positive in all the cases where culture was positive.

Table17: ANALYTICAL SENSITIVITY AND SPECIFICITY OF CONVENTIONAL METHODS FOR TRICHOPHYTON DETECTION

Method	**Sensitivity**	**Specificity**
KOH with DMSO	59%	53.7%
CULTURE	56.3%	50%

Sensitivity of the conventional methods - KOH and culture are 59% and 56.3% respectively. Specificity of both methods are 56.3% and 50% respectively. KOH has been observed to be more sensitive and specific than culture.

Table 18: ANTIFUNGAL SUSCEPTIBILITY OF DERMATOPHYTES

Dermatophytes	ITRACONAZOLE (32-0.0313µg)		TERBINAFINE (32-0.0313µg)		KETOCONAZOLE (32-0.0313µg)		FLUCANAZOLE (64-0.0625µg)		GRISEOFULVIN (32-0.0313µg)	
Isolates	MIC 50	MIC 90	MIC 50	MIC 90	MIC 50	MIC 90	MIC 50	MIC 90	MIC 50	MIC 90
T.rubrum (18)	0.125	0.25	0.0313	0.0313	0.12	0.25	16	32	0.12	0.25
T.mentagrophytes (8)	0.06	0.125	0.0313	0.0313	0.06	0.25	8	16	0.03	0.25
T.tonsurans (6)	0.03	0.125	0.0313	0.0313	0.03	0.12	8	16	0.06	0.12
T.verrucosum (5)	0.03	0.125	0.0313	0.0313	0.06	0.25	8	16	0.06	0.12
T.schonleinii (8)	0.03	0.25	0.0313	0.0313	0.06	0.25	8	16	0.06	0.12

MIC range of dermatophytes are as follows itraconazole:0.03-0.125µg, terbinafine:0.0313µg, ketaconazole:0.0313-0.25µg, flucanozole: 8-32µg, griseofulvin: 0.03-0.25µg.

TABLE 19:ANTIFUNGAL SUSCEPTIBILITY OF NONDERMATOPHYTES

Nondermatophyte fungi		Antifungal drug					
Isolate	**Nos**	**Amphotericin B(32-0.0313µg)**		**Itraconazole (32-0.0313µg)**		**Voriconazole (32-0.0313µg)**	
		MIC 50	MIC 90	MIC 50	MIC 90	MIC 50	MIC 90
Fusarium	12	1	2	16	32	8	16
Acremonium	2	0.5	0.5	8	8	4	4
Exophiala	1	1	1	0.5	0.5	0.0625	0.0625
Sytalidium	1	0.5	0.5	0.5	0.5	0.0625	0.0625
Cladosporium	2	0.5	0.5	0.5	0.5	0.0625	0.0625
Syncephalastrum	2	0.0625	0.0625	0.0625	0.0625	0.0313	0.0313
Scopulariopsis	1	0.0625	0.0625	0.0625	0.0625	0.0313	0.0313

Trichosporon	2	0.12	0.12	0.0625	0.0625	0.0313	0.0313
Geotrichum	2	0.0625	0.0625	0.0313	0.0313	0.0313	0.0313
Rhizopus	1	0.5	0.5	16	16	8	8
A.niger	2	0.25	0.25	0.0313	0.0313	0.0313	0.0313
A.flavus	1	0.25	0.25	0.0313	0.0313	0.0313	0.0313
Paecilomyces	2	0.25	0.25	0.0625	0.0625	0.0313	0.0313

MIC range of non dermatophytes are as follows : amphotericin B: 0.0625-2µg, itraconazole:0.03-32µg, voriconazole:0.0313-16µg, Fusarium and rhizopus show high MIC of 16µg for itraconazole and 8µg for voriconazole.

Table 20: **ANTIFUNGAL SUSCEPTIBILITY OF CANDIDA**

ANTIFUNGAL SUSCEPTIBILITY OF CANDIDA.	**Flucanozole (64_0.06)µg**		**Ketaconazole (32_0.03) µg**		**Itraconazole (32_0.03)µg**		**Amphotericin (32_0.03)µg**	
	MIC 50	MIC 90	MIC 50	MIC 90	MIC 50	MIC 90	MIC 50	MIC 90
C.albicans	0.5	0.5	0.25	0.5	0.o625	0.125	0.0625	0.0625
C.parapsilosis	0.5	0.5	0.25	0.25	0.0625	0.0625	0.0625	0.0625
C.tropicalis	0.5	0.5	0.25	0.25	0.0625	0.0625	0.0625	0.0625

MIC of itraconazole and amphotericin B is 0.0625µg each respectively; for ketaconazole it is 0.25µg and for flucanozole it is 0.5µg.

Table 21: COMPARISON OF E TEST AND MICROBROTH DILUTION FOR AMPHOTERICIN B

ISOLATES	**E TEST(µG)**			**MICROBROTH(µg)**		
	MIC RANGE	MIC 50	MIC 90	MIC RANGE	MIC 50	MIC 90
A.niger	0.25	0.25	0.25	0.25	0.25	0.25
A.flavus	0.25	0.25	0.25	0.25	0.25	0.25

Rhizopus	0.25	0.25	0.5	0.5	0.5	0.5
Paecilomyces	0.5	0.5	0.25	0.25	0.25	0.25
Fusarium	1-2	1	2	1-2	1	2

The MIC of *Aspergillus* and Paecilomyces are 0.25µg each respectively by both methods. MIC of Rhizopus is 0.5µg by microbroth and 0.25µg by E test method. MIC of *Fusarium* is 2µg by both the methods. Both the methods have 90% level of agreement.

Table 22: FUNGAL ETIOLOGY OF NAIL CHANGES

TOTAL CASES	**POSITIVITY RATE (CULTURE)**	**PERCENTAGE**
150	77	51.33%

The present study confirmed that diagnosis of onychomycosis based on nail changes alone may often be misleading since only 51.33% of the cases of suspected onychomycosis showed positivity by culture.

DISCUSSION

Out of 150 cases, males constituted 62% and females 38%. Thus the male female ratio has been observed to be 1.6:1. The findings of Sanjeev et al have correlated well with our study. He has also reported a similar incidence of 1.6:1. [127] Ravnborg et al have reported a male preponderance with a ratio of 2:1. [123] Nilay Kantidas et al reported a male preponderance.[112] Male preponderance may be due to occupational hazards related to the nature of work, that makes them more liable to trauma. Lower incidence of female cases may due to prevalence of social stigma, resulting in cases going unreported as reported by Amar Surjushe et al.[6]

Out of 150 patients, maximum cases were observed to be between 26 – 45 yrs (41.3%). The youngest patient was 6 yrs old and the oldest patient was 74 years old. The mean age was observed to be 40.24. Standard deviation was found to be 17.64. This study is supported by the findings of Jesudanam et al who observed increased incidence of onychomycosis in patients between 25 – 40 yrs.[90] Vinod et al reported 51.43% of onychomycotic patients to be between 20 -40 yrs.[143] The increased incidence of onychomycosis in younger population is due to their exposure to occupation related trauma and

also as they are cosmetologically more conscious than older population as reported by Vinod et al.[143]

Out of 150 patients, 28.67% constituted labourers followed by farmers (26%). Among females, housewives formed the majority of patients. P value was noted to be <0.001 for labourers and farmers which have a significant association with onychomycosis. Amar Surjushe et al reported similar increased incidence among labourers(32%) and farmers(28%).[6] Mudita Gupta et al reported 20% of patients to be labourers.[105] Labourers and farmers were more affected due to nail trauma as a result of manual labour and farming which leads to inoculation and growth of fungi causing onychomycosis as reported by Cribier et al.[29] Such increased incidence is due to increased perspiration, and greater risk of occupation related trauma and greater risk of exposure to soil saprophytes.

Regarding the comorbid conditions, 54 (36%) patients gave history of trauma while 30(20%) had diabetes mellitus and 8(5%) gave a history of immunosuppressive conditions. Trauma (P value ≤0.01) was found to be significantly associated with onychomycosis unlike other comorbid conditions which were not found to be significantly associated with onychomycosis. This correlates with the findings of Amar Surjushe et al who reported trauma in 44.66%, of patients with

onychomycosis.[6] This can explain the greater risk of occupation related trauma leading to increased exposure to soil saprophytes, thereby increasing the prevalence of onychomycosis.

In our study, 64% patients had distal lateral subungual onychomycosis (DLSO), 21.33% had total dystrophic onychomycosis (TDO), 10% had superficial white onychomycosis (SWO) and 4.6% had proximal subungual onychomycosis (PSO). Sanjeev et al reported DLSO in 82%, TDO in 5%, SWO in 2% and PSO in 1% patients.[127] Herranz et al reported DLSO in 47.61%, TDO in 28.57%, PSO in 19.04% and SWO in 4.76% patients.[77] Our study correlates well with the findings of the above studies all of which have reported a high incidence of DLSO and followed by TDO.

Among 150 patients, 54.66% had finger nail involvement, 36% had toe nail involvement and 9.33% had involvement of both nails. This correlates with the findings of Sanjeev et al who reported finger nail involvement in 48%, toe nail involvement in 30% and involvement of both nails in 22% patients.[127] Mudita Gupta et al reported finger nail involvement in 56.9% and toe nail involvement in 32.3%.[105

Increased incidence in finger nails is due to occupation related trauma and patients' concern which also lead to seeking medical attention for cosmetic purposes earlier as reported by Vinod et al.[143]

Out of 150 cases, culture was positive in 77 patients (51.33%). Among the isolates recovered, dermatophytes seem to predominate 45(54.87%); non dermatophytes were cultured from 27(35.06%) and *Candidal i*nfection was seen in 5(6.02%) patients. The predominance of dermatophytes is due to the broad spectrum of activity of the secreted proteases, which also acts as a virulence factor for nail invasion.[93] The findings in different studies are as given in the following table:

Study	**Dermatophytes**	**Nondermatophytes**	**Candida**
Pramith Ghosh(112)	50%	22.72%	-
Fragner et al (142)	65%	6.3%	18%
Walsh et al (145)	56%	11%	33%
Veer et al(143)	58%	14%	5.67%
Present study	54.87%	35.06%	6.02%

In all the above studies, dermatophytes were seen to predominate over nondermatophytes[145] which is similar to our study. The increased incidence of dermatophytic onychomycosis is due to its ability to adapt to hard keratin of the nail. The incidence of onychomycosis due to nondermatophytic mould has been variably reported in different epidemiological studies. In North America it is 4.3%, South America 4.5%[68], Columbia 9.5%[68], Argentina 1%[147], Singapore 12%[73] and

India 22%[67]. The Nilay et al reported 22.72% of nondermatophytic onychomycosis[112] and Walsh et al reported 33% of nondermatophytic onychomycosis. These recent studies suggest that the incidence of nondermatophytic onychomycosis is on the rise. These findings have also been confirmed by our study. The increasing incidence of nondermatophytic onychomycosis may be due to frequent exposure to soil saprophytes and due to expanding number of elderly and immunocompromised patients. In our study, *Candida* was isolated in 6.49% patients. This correlates with the findings of Veer et al who reported 5.67%.[143] This is in contrast to the findings of Bokhari et al who reported 46% was due to *Candida*[15]and Fragner et al who reported 18% and Walshe et al who reported *Candida* in 33% patients. In our study, among dermatophytes, *T.rubrum*(40%), *T.mentagrophytes*(17.77%), *T.schonleinii*(17.75%), *T.verrucosum* and *T.tonsurans*(11.11%) and M.audounii (2.22%) have been isolated. Nilay Kantidas et al reported *T.rubrum in* 29.54% patients. Ramesh et al reported *T.rubrum* (52%), *T.mentagrophytes* (20%), *T.tonsurans* (5.9%)[119], and Sanjeev et al reported *T.rubrum* (42.9%) *T.tonsurans* (14.3%) and *T.shonleini* (7.1%).[127] Other studies across the globe[119,15] also mentioned *T.rubrum* to be predominant etiology due to its better adaptation more virulence and easy colonization on hard

keratin (Migdley et al).[100] Shahindokht Bassiri-Jahromi et al reported *Microsporum audounii* in 1% cases.[136]

In our study *T.rubrum* (77.77%) has been predominantly isolated from DLSO type of onychomycosis. *T.mentagrophytes* was seen to be predominantly associated with PSO (62.5%). *T.verrucosum* and *T.tonsurans* were isolated in 60% cases each respectively from patients with TDO type of onychomycosis. *M.audounii* was isolated from one case suffering from DLSO type of onychomycosis. *T.schonleini* (62.5%) was isolated from patients with DLSO. Ramesh et al reported *T.rubrum* (52%) predominantly isolated from distal and lateral subungual onychomycosis (DLSO) and T.*mentagrophytes* (20%) isolated from proximal subungual onychomycosis. [119] Mudita Gupta et al reported 32% of *T.rubrum* and predominantly isolated from DLSO.[105] Sanjeev et al reported *T.schonleinii and T.tonsurans* isolated from patients having DLSO.[127] These findings correlate well with our study.

It is a well known fact that different body areas are involved by different dermatophyte species. In our study *T.rubrum* was the most frequently isolated from trunk and groin of middle aged patients. It correlates with Sanjeev et al[127] and Bassiri et al.[136] *T.mentagrophytes*

was predominantly isolated from tinea pedis; *T.tonsurans and T.shonleini* have been seen to be associated with tinea capitis. This correlates with the findings of Herranz et al[77] and Bassiri.[136] et al which shows that patients with dermatophytic skin infections are more prone for dermatophytic onychomycosis due to frequent scratching.

Diagnosis of non dermatophytic onychomycosis must be based on rigorous criteria because of their frequent presence in environment as contaminants. In our study their diagnosis is based on English and Walshe criteria. [57, 58]

Among the nondermatophytes, *Fusarium* was the most common species isolated (25.95%). *Acremonium, Cladosporium, Trichosporon, Geotrichum, Syncephalastrum, Scopulariopsis, Aspergillus niger and Paecilomyces* were positive in 7.40% cases each respectively. *Exophiala, Scytalidium, Rhizopus* and *Aspergillus flavus* were positive in 3.70% cases each respectively.In our study, *Fusarium* was the predominant species isolated. This is in contrast to Grover et al reported *A.niger* to be the predominant pathogen. [70] Immaculate xess et al reported the predominant pathogen to be *Scopulariopsis and Aspergillus fumigatus*.[79] Mathuri et al reported *Scopulariopsis* as the predominant pathogen.[100] This differs from the findings of our study.

Barde et al reported onychomycosis due to *Fusarium*.[23] Suvarchala et al reported onychomycosis due to *Exophiala*.[128] Vijaya et al reported *Trichosporon* in patients with onychomycosis.[144] Mudita Gupta et al reported *Aspergillus, Acremonium, Fusarium and Scopulariopsis*.[105] Nilay Kantidas et al[112] reported *Aspergillus niger* and Amar Surjush et al[6] reported *Cladosporium*, *Sytalidium* and Mahmoudabadi et al[97] reported *Aspergillus flavus* as primary causative agents of onychomycosis.. Nondermatophytes were isolated in significant numbers compared to dermatophytes which is consistent with Cribier's[29] findings. This could be because of environmental factors that favoured the growth of nondermatophytes, ubiquity of large and varied species of fungi in our environment as well as the active nature of our lifestyles which increases the vulnerability to trauma. It is more common in hot and humid tropical and subtropical climates according to Tosti et al[141] may be the probable cause.

Non dermatophytic molds (74.07%) were seen to be predominantly associated with DLSO. 11.11% of isolates are from total and from superficial white type each respectively and 22.22% isolates obtained from proximal subungual onychomycosis (PSO). *Trichosporon* and *Scopulariopsis* were associated with PSO; *Syncephalastrum* and

Exophiala from TDO and *Geotrichum* from SWO. Candida was isolated from DLSO(60%) and PSO(40%). This correlates with the findings of Jouni Issakainen [91] et al who reported similar findings. Nondermatophytes were more common in elderly (92.59%) (p value =0.001) and in males (81.48%) (p value =0.03). With regard to occupation farmers (66.66%) were most affected (p value =0.005) followed by labourers(18.51%). The major predisposing factors are trauma(62.96%) (p value =.0.01). The most common type was DLSO (62.96%) (p value =0.013) and it was predominantly isolated from toe nails (85.19%) (p value =0.01. This correlates with the findings of Bassiri et al[136]. Bassiri was of opinion that untreated onychomycosis could be a dangerous portal of entry for deep seated and disseminated mycosis that are hard to treat in immunocompromised patients. In our study it is more common in elderly males due to increased trauma with age, footwear, due to slow growth of finger and toe nails and circulatory disturbances. The nondermatophytes which live on unkeratinised intercellular cement may take advantage of partial denaturation of nail keratin by preexisting trauma or disease. *Candida* was isolated from 5 (6.49%) cases. Among *Candida,* 60% of isolates are *C.albicans* and 20% are *C.tropicalis* and *C.parapsilosis*. These findings correlates well with those of Kaur et al [121] and Vinodh

Sujatha[143] et al who reported that 70% of cases are due to *C.albicans.* Other *Candida* species named by them include *C.parapsilosis, C.glabrata and C.tropicalis*. All the *Candida* species (80%) were isolated from finger nail of housewives who are exposed to heavy wet work load as a result of which the growth of yeast flourishes as reported by Mudita Gupta et al. [105]

From the results obtained by direct microscopy and culture overall performance were obtained. KOH (potassium hydroxide mount) with DMSO positivity rate was seen to be 57.33%, while culture positivity rate was 51.33%.. In 86 positive direct microscopy cases 45 dermatophytes were isolated and among the remaining 41 cases 27 yielded nondermatophytes; 5 *Candida* and 9 samples (10.47%) failed to grow.KOH WITH DMSO is more sensitive than culture as reported by Mudita et al (105). Feuilhade et al[63] concluded that direct microscopy should be coupled with fungal culture for accurate diagnosis and correct species identification.

Regarding detection of dermatophytes sensitivity of conventional methods like KOH and culture are 59% and 56.3% respectively. Specificity of both methods are 56.3% and 50% each respectively. KOH is more sensitive and specific than culture. It was positive in all the culture positive cases. Additionally, it was also positive in 9 cases

which were negative by culture. None of the KOH negative cases were positive in culture. KOH mount was also negative in 64 cases which were negative by culture. These findings correlate well with those of Mudita gupta et al[105] whose KOH with DMSO and culture sensitivity rates were 59.2% and 37.6% respectively. Davies et al reported KOH sensitivity was 40% and culture 39%.[39] Direct microscopy is important for clinical diagnosis but culture required to identify the pathogenic fungi.[9] Direct microscopy positive cases are culture negative since in dystrophic nails fungus would have penetrated deep inside the matrix so that the samples from free edge of the nail may contain nonviable mycelium . Such a case will not yield a positive culture even though microscopy is positive. This opinion is also shared by David et al.[39].

It is essential that good lab methods are available for rapid and precise identification in order to apply appropriate treatment. The conventional methods have their own drawback. KOH with DMSO has low specificity; culture has low sensitivity and also takes longer time. The changing profile of human dermatoses has created a need for improved diagnostic methods. So newer diagnostic methods are need of the hour not only for accurate diagnosis but also for post therapeutic strategies. The application of PCR directly to clinical

samples will help to detect the causative agent and help in early initiation of treatment. The present study tested such a direct PCR from clinical samples for the detection of *Trichophyton* genus which is the predominant causative agent of onychomycosis. In general identification method based on genotype of an isolate is far better than direct microscopy and culture. Our study targets the internal transcribed spacer region 1 of trichophyton 18srRNA gene (ITS 1)200bp.

In our study we compared direct microscopy, culture and PCR from 117 clinical samples (after excluding nondermatophyte culture positive cases, *Candida* and Microsporum audouni) for detection of *Trichophyton* genus. Among the remaining 117 cases, KOH mount was observed to be positive in 53 cases (45.30%), culture in 44cases (37.61%) and PCR in 66 cases (56.41%). All the 44cases which were positive by KOH and culture were also positive with PCR. Out of 9 isolates which were positive by KOH mount and negative by culture, 7 shown to be positive with PCR and two of them are negative. Additionally 13 cases which were negative by both KOH mount and culture were positive by PCR. The remaining 51 cases which were negative by both KOH mount and culture were also seen to be negative with PCR. This could be due to that the material was not

being hydrolyzed long enough and inadequate time spent in examination. It correlates with the findings of Kardjeva et al [93] who reported that KOH, culture and PCR were positive in 64%, 22%, and 84% cases respectively. Garg et al reported positivity rate of KOH, culture and PCR to be (70%, 25.8%, 50.8% respectively in first round and 83.8% in second round. The target was chitin synthase gene. [84] In the study by Brillowska, he described the sensitivity of KOH and culture to be 22.92% each respectively and PCR to be 41.5%. PCR was positive in 100% of cases where culture was positive. [26]

Analytical sensitivity and specificity of PCR were calculated by means of using ATCC *T.rubrum* 34265. The analytical sensitivity of the primer was 50 copies /ml of ATCC strain. The primers were specific such that it can amplify all the *Trichophyton* isolates tested and not amplifying the other nondermatophyte fungal DNA.

When compared with the conventional method the positivity rate of this PCR assay is strongly statistically significant ($P<0.003$) by using Chi square test. Our findings relate well to recent studies by various groups that report successful and rapid demonstration of *Trichophyton* genus in nails by using molecular methods. The current disadvantage of PCR is its higher cost and it need laboratory equipment but an

accelerated finding of a final diagnosis can considerably reduce treatment cost.

The treatment of onychomycosis consists of prolonged course of antifungals and since onychomycosis in chronic immunosuppressed patients can be a reservoir of infection appropriate antifungals have to be prescribed. So in this study susceptibility testing was done for dermatophytes, filamentous fungi and yeast according to M 27- P ,M38- Pand M- 38 A of NCCLS criteria.

MIC range of itraconazole for all dermatophytes was 0.03-0.125µg. MIC range of terbinafine for all dermatophytes was 0.0313 µg. MIC range of ketaconazole for all dermatophytes are 0.0313-0.25µg. MIC range of flucanozole for all dermatophytes was 8-32µg. MIC range of griseofulvin for all dermatophytes was 0.03-0.25µg. In our study the evaluation of tested drugs reveal that terbinafine was the most potent drug (statistically significant with $P \leq 0.01$) for dermatophytes confirming reports by Kortind et al and Santos et al.[13] Among azoles itraconazole was the most potent drug (statistically significant with $P<0.01$) followed by ketaconazole and flucanozole. These findings were supported by those f Hamdan et al and Korting et al.[12,13] With respect to griseofulvin majority of the isolates have 0.0625µg as reported by Jessup et al.

Drugs	Present study			Dr. Pankajalakshmi Study			M.A.Ghannoum et study		
	MIC RANGE (µg/ml)	**MIC 50 (µg/ml)**	**MIC 90 (µg/ml)**	**MIC RANGE (µg/ml)**	**MIC 50 (µg/ml)**	**MIC 90 (µg/ml)**	**MIC RANGE (µg/ml)**	**MIC 50 (µg/ml)**	**MIC 90 (µg/ml)**
Griseofulvin	0.03-o.25	0.06	0.12	0.1-10	1	5	-	-	-
Ketoconazole	0.0313-0.25	0.06	0.25	0.01-5	1	2.5	0.12-64	0.12	0.5
Flucanazole	8-32	8	16	-`	-	-	0.12-16	2	16
Itraconazole	0.03-0.125	0.03	0.125	0.01-0.5	0.1	0.5	0.001-0.05	0.015	0.125
Terbinafine	0.0313	0.0313	0.0313	0.001-0.01	0.01	0.1	0.001-0.5	0.008	0.03

All the above studies show that terbinafine is most effective (statistically significant with $P \leq 0.01$) followed by itraconazole, griseofulvin, ketocanozole and finally flucanozole. Our study correlates well with Dr.Pankajalakshmi study and M.A.Ghannoum et al[67] except that the MIC of flucanozole is higher for our isolates of a range of 8-32 µg. This variability is due to important methodological difference among laboratories.

In this study we compared the activity of three antifungals against nondermatophytes Among nondermatophytes MIC range of amphotericin B for filamentous fungi was 0.0625-2µg. MIC range of itraconazole for nondermatophytes was 0.03-32µg. MIC range of voriconazole was 0.0313-16µgl. Among the azoles voriconazole was potent than itraconazole as confirmed by Espinel et al[61,67] and for

Fusarium and *Rhizopus* whose voriconazole MIC are higher by about 8µg as reported by Sekely et al[2[and are not effective.[30] MIC of itraconazole was higher than voriconazole for *Fusarium and Rhizopus* of about 32µg as reported by Denning et al [47].This is also confirmed by Espinel Ingroff[61] et al . Amphotericin B was effective against all isolates except *Fusarium* whose MIC 90 value is 2µg supported by the studies done by Dannaoui et al[59] and Espinel ingroff et al.[61] In conclusion voriconazole was statistically significant ($P<0.05$) than itraconazole against all isolates except *Rhizopus* and *Fusarium* where azoles were not effective. Amphotericin B whose MIC 90 (0.5µg) is effective and statistically significant $P\leq0.01$ against all isolates except *Fusarium* whose MIC 90 is 2µg.

Among *Candida* all were sensitive to amphotericin B and itraconazole equally with a MIC of 0.0625µg. MIC of ketaconazole is 0.125µg and for flucanozole is higher of 1µg.Regarding *Candida,* amphotericin B and itraconazole were more effective than ketaconazole and flucanozole. But statistically the difference was not significant ($P>0.01$). Among the *Candida* species all were equally sensitive. This correlates with the findings of Espinel Ingroff et al.[61] The microdilution assay for dermatophytes and filamentous fungi and

yeast are convenient and reproducible. The result of this method showed good level of agreement between other studies.

Since the NCCLS methods are time consuming, E test is an alternative approach for the antifungal susceptibility testing for filamentous fungi. In this study we evaluated activities of amphotericin B by E test method. The MIC of *Aspergillus* was found to be 0.25µg by both methods. MIC of *Rhizopus* and *Paecilomyces* were 0.5µg and 0.25µg respectively by microbroth dilution method and 0.25µg and 0.5µg respectively by E test method. MIC of *Fusarium* was found to be 2µg by both the methods. MIC determined by E test and microbroth dilution showed > 90% level of agreement. Since the E strip contains a continuous gradient instead of the established twofold drug dilution scheme the exact concentration can be determine. Our results show that E test is reproducible as reported by Szekely et al[2] and Wanger[9] et al. This demonstrated that it is less labour intensive and much simpler to set up than the broth dilution.

The present study confirmed that the diagnosis of onychomycosis based on nail changes alone may often be misleading since only 51.33% of the cases of suspected onychomycosis showed positivity by culture. Other workers also reported similar results of 43.7% in Poland[98], 50.6% in Turkey[90], 45.53% and 51.76% in India.[112] This

shows that all the nail changes need not necessarily be of fungal origin as onychomycosis may also mimic other disease like psoriasis and lichen planus. Here we emphasise the need to obtain definitive diagnosis of fungal infection before initiation of antifungal therapy in case of dystrophic nails.

SUMMARY

150 clinically diagnosed cases of onychomycosis attending the mycology unit of Dermatology outpatient department of Stanley medical college between OCTOBER 2010 to AUGUST 2011 were included in the study. Nail samples collected were split equally and were subjected to KOH with DMSO, culture and PCR.

1. The prevalence rate of onychomycosis in clinically diagnosed cases is 51.33%.

2. Male female ratio is 1.6:1. 41.3% of cases were aged between 26-45 years.

3. Increased incidence was among labourers (28.67%) and farmers (26%).

4. Among the comorbid conditions, 36% gave history of trauma, while 20% had diabetes mellitus and 5.33% had immunosuppressive diseases.

5. Culture was positive in 51.33%. Among the isolates recovered, dermatophytes were seen to predominate (58.44%). Nondermatophytes were cultured from 35.06% cases and candidal infection was seen in 6.49%.

6. Among the dermatophytes, *T.rubrum* was the predominant species isolated (40%). T.mentagrophytes (17.77%) and T.schonleinii (17.77%) were the next most common species isolated. T.verrucosum and T.tonsurans were in 11.11% cases each respectively. Microsporum audounii was isolated from 2.22% cases.

7. T.rubrum (77.77%) has been predominantly isolated from DLSO type of onychomycosis. *T.mentagrophytes* (62.5%) was seen to be predominantly associated with PSO type of onychomycosis.

8. Among the nondermatophytes, Fusarium was the most common species isolated (25.95%). *Acremonium, Cladosporium, Trichosporon, Geotrichum, Syncephalastrum, Scopulariopsis, Aspergillus niger* and *Paecilomyces* were positive in 7.40% cases each respectively. *Exophiala, Scytalidium, Rhizopus* and Aspergillusflavus were positive in 3.70%cases each respectively.

9. Non dermatophytic molds (74.07%)were seen to be predominantly associated with DLSO. 11.11% of isolates were isolated from TDO and SWO type of onychomycosis; 2.22% from PSO type of onychomycosis.

10. Candida was isolated in 5 (6.49%) of cases. Among them, *C.albicans* (60%)was the predominant species isolated. Other species

isolated include *C.tropicalis* (20%) and *C.parapsilosis* (20%). Candida was isolated from DLSO(60%) and PSO(40%).

11. KOH with DMSO positivity for all cases was observed to be 57.33% and culture positivity 51.33%.

12. Among the remaining 117 cases, KOH with DMSO mount positivity was observed to be positive in 45.30%, culture in (37.60%) and PCR in (56.41%).

13. Sensitivity of conventional methods like KOH with DMSO and culture are 59% and 56.3% respectively. Specificity of both methods are 53.7 and 50% respectively. KOH has been observed to be more sensitive and specific than culture.

14. In our study the evaluation of tested drugs reveal that terbinafine($P < 0.05$) was the potent active drug for dermatophytes.

15. For treatment of nondermatophytes, voriconazole was more effective (statistically significant with $P<0.05$) than itraconazole against all isolates except *Rhizopus* and *Fusarium* where azoles were not effective

16. Among Candida all species were sensitive to amphotericin - B and itraconazole equally with a MIC of 0.0625µg and to ketaconazole with an MIC of 0.125µg. The MIC for flucanozole is higher of 1µg.

17. MIC values determined by E test and microbroth dilution showed good level of agreement. Our results show that E test is reproducible and easier to perform.

CONCLUSION

The nail changes are not always a reliable marker for predicting the causative organism, and relying only on the clinical manifestation in the diagnosis is often misleading since only 51.33% of the suspected case of onychomycosis showed positivity by culture. Knowing the exact pathogen is important and has implication in therapy and prognosis. Our study highlights the need for microbiological confirmation in case of onychomycosis.

There is a relatively high incidence of fingernail onychomycosis of DLSO pattern in middle aged men. Although regional and temporal variability exists among the microorganisms that are pathogenic in onychomycosis, dermatophytes still remain the predominant etiological agents. Terbinafine has been observed to be the potent drug active against dermatophytes.

The environmental ecology changes from time to time and nondermatophytes which were previously considered as contaminants are now emerging as primary pathogens and their definitive diagnosis can be done only on the basis of stringent criteria .

The non dermatophytes are responsible for 35.06% of cases of onychomycosis. Voriconazole and amphotericin - B have been

observed to be effective against nondermatophytes. Candida albicans are more common among housewives who were exposed to heavy wet workload which helps the yeast to flourish.

Direct microscopy and culture are necessary for all suspected cases and can be used in a laboratory with a minimal infrastructure. Each laboratory should set up consecutive cultures from consecutive nail scrapings. Among conventional methods KOH with DMSO is more sensitive than culture.

The changing profile of human dermatoses has been observed of late which requires improved diagnostic methods for identification. So newer diagnostic methods are need of the hour not only for accurate diagnosis but also for post therapeutic strategies. The application of PCR directly to clinical samples along with conventional methods will help to detect the causative agent early and accurately and helps not to miss other fungi which would permit prompt and targeted intiation of antifungal therapy.

The E test can be considered a good alternative. In addition, education of patients about the importance of foot and nail care and earlier treatment of dermatophytic skin infections should be considered as essential components in the management of these patients.

APPENDIX

Sabouraud Dextrose Agar with Antibiotics
Composition of Sabouraud's Dextrose Agar (Emmons Modification)

20 gm	:	Dextrose
10 gm	:	Peptone
20 gm	:	Agar
1000 ml	:	Distilled Water
6.9	:	Final pH

The ingredients by dissolved by boiling 50 milligram per Ltr. of chloramphenicol was added and 500 milligrams per Ltr. of Cycloheximide, Chloramphenicol was dissolved in 10 ml of 95% Ethanol and added to boiling medium. Chloramphenicol was dissolved in 10 ml of acetone and added to the boiling medium. Autoclave 150^0 for 15 minutes, Dispense in Tubes and allow to Cool in slanted position.

Brain – Heart Infusion Agar
Composition

37 g,	:	Brain Hear Infusion
20 gm	:	**Glucose**
1 gm	:	L-Cysteine Hydrochloride
20 gm	:	Agar
900 ml	:	Distilled Water

Dissolve ingredients by boiling. Dispense into Screw-captubes and auto elevate 121^0 C for 15 minutes. The antibiotics that iscyclohexinride, chloramphenicol and gentamicin are added to this medium as for SDA. Cool is slanted position stored in refrigerator pH is adjusted to 6.7.

BLOOD AGAR

It contains the following ingredients :

40 gm : Agar based

50 ml : Sheep blood

100 ml : Distilled water

MODIFIED CHRISTENSEN'S MEDIUM FOR UREASE

HYDROLISIS :

1.0 g : Peptone

5.0 g : NaCl

2.0 g : $KH_2 PO_4$

5.0 g : Glucose

20.0 g : Agar

1000 ml : Distilled water

After dissolving the above ingredients by heat 5 ml of phenol red solution (0.2% in 50% alcohol) was added after which autoclaved at 115^0 Centigrade per 15 minutes. On cooling 100 ml of urea (20% aqueous solution sterilized by solution) was added medium was poured into slopes.

POTATO DEXTROSE AGAR

For convenience this medium is generally prepared from dehydrated commercial preparation according to the instruction of the manufacturer. Alternatively this medium may be prepared from raw materials as follows:

200 g : Potato

20 g : Dextrose

20 g : Agar

1 Lit : Water

Scrub but do not peel the potato & cut into 12 ml cubes, boil 200g in 1 litre of water for 60 minutes.

Squeeze as much of the pulp as possible through a fine sieve. Add agar and boiled till dissolved. Add dextrose and make up to 1 litre. Dispense in required amounts taking care to keep solids in suspension. Autoclave at 115^0 C for 30 minutes. Cool to 50^0 C & pour into tubes and allowed to cool in slanted position.

POTASSIUM HYDROXIDE MOUNTS

It is prepared from the following ingredients

10 gms : Potassium hydroxide

10 ml : Glycerol

80 ml : Distilled water

To a solution of 10% KOH, 10% Glycerol is added to prevent drying-Nix ingredients and store at room temperature.

LACTOPHENOL COTTON BLUE STAIN

The lacto phenol cotton blue (LCD) is used to study the morphological features of the fungal isolates. It is of two types :

Plain LCB (i)

It contains the following ingredients:

20 ml : Melted phenol

20 ml : Lactic acid

40 ml : Glycerol

0.05 gm : Cotton blue

20 ml : Distilled water

Mix all the reagents properly and dissolve 0.05 g of cotton blue stain in the distilled water before mixing with the remaining reagents. The phenol acts as disinfectant, Lactic Acid preserves the morphology of the fungi and glycerol is hygroscopic agent which prevents drying. The cotton blue stains the outer wall of the fungus. Tease out of a fragment of the culture on a glass slide in a drop of LCB using two teasing needles. Put of a coverslip and examine under the microscope. If the plane LCB is used the edges of the coverslip can be sealed with nail polish to keep it for longer period of time.

Table 1:

Scheme for preparing dilution series of water-insoluble antifungal agents to be used in broth dilution susceptibility tests

Antimicrobial solution						
Step	**Concentration (µ.g / mL)**	**Source**	**Volume + Solvent (mL) e.g. DMSO**	**= Intermediate concentration (µ.g / mL)**	**= Final Concentration at 1:50 (µ.g / mL)**	**Log**
1	1600	Stock		1600	32	4
2	1600	Stock	0.5 0.5	800	16	3
3	1600	Stock	0.5 1.5	400	8	2
4	1600	Stock	0.5 3.5	200	4	1
5	200	Step 4	0.5 0.5	100	2	0

6	200	Step 4	0.5 1.5	50	1	-1
7	200	Step 4	0.5 3.5	250	0.5	-2
8	25	Step 7	0.5 0.5	12.500	0.25	-3
9	25	Step 7	0.5 1.5	6.25	0.125	-4
10	25	Step 7	0.5 3.5	3.13	0.0625	-5

Table 2: Scheme for preparing dilution of water-insoluble antifungal agents to be used in broth dilution susceptibility tests

Antimicrobial solution						
Step	**Concentration (µ.g / mL)**	**Source**	**Volume + Medium (mL)(mL)**	**= Intermediate concentration (µ.g / mL)**	**= Final Concentration at 1:50 (µ.g / mL)**	**Log**
1	5120	Stock	7 1	640	128	6
2	640	Step 1	1.0 1	320	64	5
3	640	Step 1	3.0 1	160	32	4
4	160	Step 3	1.0 1	80	16	3
5	160	Step 3	1.5 0.5	40	8	2
6	160	Step 3	3.5 0.5	20	4	-1
7	20	Step 6	1.0 1	10	2	-0
8	20	Step 6	1.5 0.5	5	1	-1
9	20	Step 6	3.5 0.5	2.5	0.5	-2
10	2.5	Step 9	1.0 1	1.25	0.25	-3
11	2.5	Step 9	1.5 0.5	0.625	0.125	-4
12	2.5	Step 9	3.5 0.5	0.3125	0.0625	-5

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MASTER SHEET

DEPT OF MICROBIOLOGY

GOVT STANLEY MEDICAL COLLEGE

S.NO	NAME	AGE	SEX	OP/IP NO	OCCUPATION	SKIN INV	TRAUMA	DIA/IM,S	FN/TN	TYPE	KOH	CULTURE	PCR	mic ter	mic gri	mic ket	mic flu	mic itr	mic vori	mic amb
1	Sengayyan	27	M	698473	LABOUR	_	_	_	fn	DLSO	_	_	_							
2	angappan	55	M	703270	FARMER	_	+	_	tn	TND	_	_	_							
3	Sagunthala Devi	21	F	703208	STUDENT	_	+	_	TN	DLSO	+	FUSARIUM						16	8	2
4	Yashodha	27	F	692179	OFFICE	_	_	_	FN	TND	_	_	_							
5	Meerat	6	M	692124	STUDENT	+	_	_	FN	DLSO	_	_	+							
6	RENGAN	33	M	761527	OFFICE	_	_	DIA	FN	DLSO	_	_	_							
7	Kishore Kumar	60	M	698052	LABOUR	_	_	-	FN	TND	_	_	_							
8	Kumar	14	M	692506	STUDENT	_	_	_	fn	DLSO	+	_	+							
9	Athilakshmi	33	F	703349	OFFICE	+	_	_	fn	DLSO	+	T.RUBRUM	+	0.03	0.25	0.25	0.5			
10	Alagarsamy	33	M	743124	LABOUR	_	_	_	FN	DLSO	_	-	-							
11	Kali	60	M	628461	FARMER	_	+	_	FN	DLSO	_	_	+							
12	Parani	17	M	628462	STUDENT	+	+	_	FN	DLSO	+	T.VERRUCOSUM	+	0.03	0.25	0.25	0.5			
13	Vadivelu	44	M	628475	LABOUR	+	_	DIA	FN/TN	DLSO	_	_	+				0			
14	Sheeba	30	F	613421	OFFICE	_	_	_	FN	PSO	_	_	_							
15	Ramakrishnan	32	M	627136	LABOUR	_	_	DIA	FN	DLSO	+	_	_							
16	SUMAN	23	M	702305	STUDENT	+	_	_	TN	DLSO	+	T.VERRUCOSUM	+	0.03	0.25	0.5	0.5			
17	Sahina	33	F	702431	OFFICE	_	_	_	FN	TND	_	_	+							
18	RAJI	18	F	704321	STUDENT	_	_	_	FN	DLSO	+	T.MENT	+	0.03	0.25	0.25	0.5			
19	Ram	38	F	716201	FARMER	_	_	_	FN	DLSO	+	T.RUBRUM	+	0.03	0.12	0.12	0.25			
20	Kannan	18	M	714362	STUDENT	_	_	_	FN	SWO	_	_								
21	Ibrahim	30	M	714316	LABOUR	_	_	_	FN	DLSO	_	_	+							
22	chellam	65	M	701432	LABOUR	+	_	_	FN	DLSO	+	T.VERRUCOSUM	+	0.03	0.25	0.25	0.5			
23	Selvi	30	F	681403	lABOUR	_	+	_	tN	DLSO	+	CLADOSPORIUM						0.625	0.03	0.03
24	Ayyammal	32	F	681404	HOUSEWIFE	_	_	_	FN	DLSO	_	_	+							
25	Rani	29	M	681413	LABOUR	+	_	_	FN	TND	+	T.SCHONLEINI	+	0.03	0.12	0.25	0.5			
26	DHANASEKAR	50	M	701414	LABOUR	__	_	_	FN	DLSO	_	_	_							
27	Renu	62	M	731461	FARMER	_	+	_	TN	DLSO	+	FUSARIUM						16	4	0.5
28	Abdul Hakkim	20	M	724120	LABOUR	_	_	_	FN	SWO	_	_	+							
29	Murugan	35	M	701447	LABOUR	+	_	_	FN	DLSO	+	T.RUBRUM	+	0.03	0.12	0.25	0.5			
30	Radha	13	F	701448	STUDENT	_	_	_	FN_	TND	_	_	_							
31	Nagarathinam	60	M	701449	FARMER	_	+	_	FN	TND	_	_	_							
32	Rathinam	25	F	701450	HOUSEWIFE	+	_	_	FN	DLSO	+	T.MENT	+	0.03	0.06	0.12	0.25			
33	Abama	61	M	701452	LABOUR	_	_	DIA	TN	DLSO	+	FUSARIUM						4	2	0.5
34	Banumathi	54	F	701453	FARMER	+	_	_	fn	DLSO	+	T.TONSURANS	+	0.03	0.03	0.62	0.12			
35	HARI	28	M	701454	OFFICE	_	_	DIA	FN	SWO	_	_	+							
36	KALA I	24	M	701455	LABOUR	+	_	_	FN	DLSO	+	T.TONSURANS	+	0.03	0.62	0.06	0.25			
37	Veena	26	F	701456	OFFICE	_	_	IS	TN	DLSO		ACREMONIUM						4	2	0.5
38	Guru	30	M	701112	FARMER	_	+	_	TN	DLSO	+	SYNCEPHALASTRUM						0.12	0.06	0.06

39	PANDU	10	M	701143	STUDENT	_	+	_	TN	DLSO	_	_	_							
40	Shamima	69	M	701431	LABOUR	_	+	_	FN	TND	+	T.SCHONLEINI	+	0.03	0.12	0.06	0.5			
41	Shakunthala	33	M	701442	LABOUR	_	+	_	FN	DLSO	+	T.RUBRUM	+	0.03	0.06	0.12	0.25			
42	Rajasekhar	25	M	701431	LABOUR	_	_	_	TN	DLSO	_	_	_							
43	Fathima	23	F	701421	HOUSEWIFE	_	_	_	FN	TND	_	_	+							
44	Ashraf	60	M	703142	FARMER	_	+	DIA	TN	DLSO	+	RHIZOPUS						4	2	0.5
45	JEEVA	30	M	703415	LABOUR	+	_	+	FN	SWO	+	T.MENT	+	0.03	0.12	0.06	0.5			
46	Jeya	25	F	703221	LABOUR	_	+	_	TN	DLSO	+	FUSARIUM						8	4	0.06
47	Rajeshwari	27	F	701642	LABOUR	+	_	_	FN	TND	+	T.SCHONLEINI	+	0.06	0.03	0.06	1			
48	Kannayan	44	M	701234	LABOUR	_	_	_	FN	DLSO	+	T.RUBRUM	+	0.03	0.06	0.12	0.5			
49	Unni	8	M	703100	STUDENT	+	_	_	FN	DLSO	_	_	+							
50	Ambujam	50	F	704124	HOUSEWIFE	+	_	DIA	FN	TND	+	T.TONSURANS	+	0.03	0.12	0.06	0.5			
51	Sujatha	26	F	701425	HOUSEWIFE	+	_	_	F	DLSO	_	_	+							
52	HARI	60	M	701432	FARMER	_	+	_	TN	DLSO	+	A.NIGER						0.5	0.25	0.06
53	MEGHAN	45	M	701440	LABOUR	_	_	_	FN/TN	DLSO	_	_	_							
54	Beera	25	M	701432	LABOUR	_	_	_	TN	SWO	_	_								
55	Dileepan	60	M	701441	LABOUR	_	_	_	TN	SWO	+	T.MENT	+	0.03	0.06	0.06	0.5			
56	mani	20	F	701442	STUDENT	+	_	_	fn	DLSO	_	_	+							
57	Girija	22	M	701342	STUDENT	_	_	_	FN	DLSO	+	T.RUBRUM	+	0.06	0.03	0.12	0.5			
58	Yashodha	59	F	701464	LABOUR	_	_	DIA	FN	TND	+	T.SCHONLEINI	+	0.06	0.03	0.25	1			
59	GOWRISHANKAR	30	M	703421	LABOUR	_	+	_	FN	DLSO	+	T.MENT	+	0.06	0.12	0.06	0.5			
60	Thangammal	20	F	706140	HOUSEWIFE	_	_	_	FN_	PSO	+	FUSARIUM						0.5	0.25	0.06
61	Rohith	40	M	704320	FARMER	_	+	_	TN	DLSO	+	CLADOSPORIUM						0.25	0.06	0.03
62	Mohan	33	M	701412	LABOUR	+	+	_	FN	TND	+	T.VERRUCOSUM	+	0.03	0.06	0.12	0.5			
63	Mariaeswaran	45	F	703460	LABOUR	_	_	_	FN	DLSO	+	T.TONSURANS	+	0.06	0.12	0.03	1			
64	Siva	32	M	703412	LABOUR	_	_	_	FN	DLSO	_	_	_							
65	Irfan	7	M	704240	STUDENT	+	_	_	FN/TN	DLSO	+	T.RUBRUM	+	0.03	0.06	0.12	0.5			
66	Ashraf	31	M	703121	LABOUR	_	_	_	FN	DLSO	+	_	+							
67	Kala	30	M	764140	FARMER	_	_	DIA	FN	DLSO	+	PAECILOMYCES						0.5	0.25	0.06
68	Rupavathy	19	F	721420	STUDENT	+	-	_	FN	DLSO	+	T.VERRUCOSUM	+	0.03	0.06	0.12	0.5			
69	MURALI	33	M	734260	LABOUR	_	+	DIA	FN	DLSO	_	_	+							
70	SEKHAR	21	M	741240	STUDENT	_	_	_	FN	PSO	+	T.MENT	+	0.06	0.03	0.12	0.5			
71	Ravichander	69	M	711421	LABOUR	_	+	IS	TN	DLSO	+	A.NIGER	+					0.5	0.25	0.06
72	DHANASEKAR	64	M	711432	LABOUR	_	+	_	FN	DLSO	_	_	+							
73	Rajeshwari	55	F	714361	FARMER	_	+	_	TN	DLSO	+	FUSARIUM						8	4	2
74	KAMALAN	54	M	702620	FARMER	_	+	_	FN	DLSO	_	_	+							
75	Ponnuthai	36	F	703410	HOUSEWIFE	+	_	DIA	FN	DLSO	+	TRICHOSPORON						8	4	2
76	UMAYAPPAN	38	M	704120	LABOUR	_	+	_	FN	DLSO	+	T.RUBRUM	+	0.03	0.06	0.06	0.5			
77	Chandran	23	M	704610	LABOUR	_	_	_	FN	SWO	_	_	+							
78	Palayammal	68	F	704431	HOUSEWIFE	_	_	_	FN	DLSO	_	_	+							
79	Salim	40	M	704321	FARMER	_	+	_	fn	DLSO	+	T.TONSURANS	+	0.03	0.06	0.12	1			
80	Shanthi	22	F	704316	LABOUR	_	+	_	TN	TND	_	_	+							

81	Dileepan	22	M	710460	LABOUR	_	+	_	fn	DLSO	+	T.RUBRUM	+	0.03	0.03	0.06	1			
82	Vasuki	26	F	711430	HOUSEWIFE	_	_	_	FN	TND	+	_	+							
83	Raja	44	M	711420	FARMER	_	_	_	TN	DLSO	+	A.NIGER						0.62	0.03	0.03
84	LAKSHMANAN	24	M	704162	LABOUR	_	_	_	FN	DLSO	_	_	_							
85	Govindasamy	29	M	703142	LABOUR	+	+	_	FN/TN	DLSO	_	_	+							
86	Pari	33	M	703155	LABOUR	_	_	_	FN	TND	_	_	_							
87	Mohan	64	M	704162	LABOUR	+	_	_	TN	DLSO	+	T.RUBRUM	+	0.03	0.06	0.12	0.5			
88	Nazeer	35	M	703124	FARMER	_	_	_	TN	DLSO	+	PAECILOMYCES						0.06	0.03	0.03
89	Abhizha	21	m	702162	STUDENT	+	_	_	fn	TND	+	T.SCHONLEINI	+	0.03	0.12	0.12	1			
90	Devaraj	48	M	703421	LABOUR	+	_	_	FN	SWO	_	_	+							
91	Ramani	60	M	741062	HOUSEWIFE	_	_	_	FN	SWO	_	_	_							
92	JOHNSON	56	F	741432	FARMER	_	_	_	TN	TND	_	_	_							
93	Kayalvizhi	28	F	701464	MISC	_	+	_	FN_	DLSO	_	_	+							
94	MARAN	50	M	703211	LABOUR	_	_	_	FN	DLSO	+	T.MENT	+	0.03	0.06	0.06	0.06			
95	Arunachalam	28	M	703241	LABOUR	_	_	_	FN	TND	_	_	_							
96	Bazeera Begum	70	M	703142	FARMER	_	_	_	TN	TND	+	T.TONSURANS	+	0.03	0.03	0.03	0.03			
97	Parvathy	33	F	702421	HOUSEWIFE	_	_	IS	FN	PSO	+	TRICHOSPORON						2	1	1
98	Krishnan	14	M	703416	STUDENT	+	_	_	FN	DLSO	_	_	_							
99	Kannan	55	M	703214	LABOUR	_	_	DIA	FN	TND	_	_	+							
100	Britto	29	M	701421	FARMER	_	_	_	FN	DLSO	+	T.VERRUCOSUM	+	0.03	0.03	0.06	0.5			
101	Latha	17	F	741642	STUDENT	_	_	IS	FN	SWO	+	GEOTRICHUM						0.06	0.03	0.03
102	Appu	56	M	724612	FARMER	_	_	_	TN	DLSO	+	ACREMONIUM						0.12	0.06	0.03
103	Katheeja Banu	35	F	734124	HOUSEWIFE	+	_	DIA	FN	DLSO	+	C.ALBICANS					0.5	0.25	0.12	0.12
104	Maheshwari	41	F	721412	HOUSEWIFE	_	_	_	FN	DLSO	_	_	+							
105	Valliammal	45	F	701612	HOUSEWIFE	_	_	DIA	TN	TND	_	_	_							
106	NALAN	54	M	703212	FARMER	_	_	IS	fn	SWO	+	FUSARIUM						0.12	0.06	0.03
107	Nambunayagi	43	F	702212	HOUSEWIFE	_	_	_	FN	DLSO	_	_	_							
108	SASI	61	M	701426	LABOUR	_	_	_	FN	DLSO	_	_	_							
109	Avama	74	F	706425	HOUSEWIFE	_	_	_	FN/TN	DLSO	+	T.RUBRUM	+	0.03	0.03	0.03	1			
110	Kumarappan	30	M	736214	FARMER	_	+	_	TN	DLSO	_	SYTALIDIUM						0.06	0.03	0.03
111	Yogalakshmi	18	F	721429	HOUSEWIFE	_	_	_	TN	TND	_	_								
112	MAHADEVAN	62	M	700421	LABOUR	+	_	_	FN	DLSO	+	_	+							
113	Vasu	11	M	736412	STUDENT	+	_	_	FN	DLSO	+	T.RUBRUM	+	0.03	0.06	0.06	1			
114	Ponmani	65	F	724141	LABOUR	_	+	_	FN	DLSO	_	_	+							
115	SEKAR	65	M	731462	LABOUR	_	_	DIA	FN/TN	TND	+	T.SCHONLEINI	TND	0.06	0.06	0.03	0.5			
116	Kaveri	34	F	731468	FARMER	+	_	_	FN	DLSO	_	_	+							
117	Kaliammal	45	F	704126	HOUSEWIFE	_	_	DIA	FN	SWO	_	_								
118	RAHIMA	68	F	721421	LABOUR	_	_	IS	FN	PSO	*+	TRICHOSPORON						4	2	0.5
119	RAJAN	23	M	736124	STUDENT	+	_	_	TN	TND	+	T.SCHONLEINI	+	0.03	0.03	0.06	0.5			
120	Thiyagarajan	73	M	741245	LABOUR	_	_	_	FN	DLSO	_	_	+							
121	HAKKIM	30	F	700016	LABOUR	_	_	DIA	TN	PSO	_	T.MENT	+	0.06	0.03	0.06	0.5			
122	Kannamma l	42	F	701431	HOUSEWIFE	_	_	DIA	FN	TDO	_	_	_							

123	Ganesh	54	F	605793	LABOUR	_	_	IS	FN_	SWO	+	A.FLAVUS						0.06	0.03	0.06
124	Nandhini	58	F	604621	FARMER	_	_	DIA	FN	DLSO	_	_	+							
125	RAVI	32	M	734125	FARMER	_	+	_	TN_	TND	+	EXOPHIALA						0.06	0.03	0.03
126	NEELAM	69	F	724122	HOUSEWIFE	+	_	_	FN	DLSO	_	_	+							
127	Sherif	25	M	734142	LABOUR	_	_	_	TN	DLSO	+	ACREMONIUM						0.06	0.03	0.03
128	Jeyalakshmi	62	F	741200	HOUSEWIFE	_	_	_	FN	TND	_	_								
129	Ambigapathy	53	m	731461	FARMER	_	_	DIA	FN	DLSO	+	T.TONSURANS	+	0.03	0.12	0.12	0.5			
130	Shantha	44	F	731621	FARMER	_	_	_	FN	DLSO	_	_	+							
131	THAMAN	72	M	704124	LABOUR	_	+	_	TN_	DLSO	_	FUSARIUM						2	1	0.5
132	Latha	22	F	736425	HOUSEWIFE	_	_	IS	TN	PSO	+	SCOPULARIOPSIS						0.12	0.06	0.06
133	MANI	68	M	714214	LABOUR	_	_	_	TN	DLSO	+	_	+							
134	Selvi	50	F	736124	HOUSEWIFE	_	_	DIA	FN	PSO	+	C.TROPICALIS					0.12	0.06	0.03 ket	0.03
135	Mariaeswaran	60	M	670421	FARMER	_	_	DIA	TN/FN	DLSO	+	T.TONSURANS	+	0.03	0.12	0.12	0.5			
136	Rajakumari	69	F	614624	HOUSEWIFE	_	_	_	TN	TDO	_	_	_							
137	Vasantha Mariammal	66	F	641642	HOUSEWIFE	+	_	_	FN	DLSO	+	T.RUBRUM	+	0.03	0.12	0.06	0.5			
138	BHARANI	25	M	631625	MISC	_	_	_	_	SWO	_	_								
139	Rathna	43	F	636125	HOUSEWIFE	+	_	_	FN	SWO	+	C.PARAP					0.06	0.03	0.03-ket	0.03
140	ARuLNATHAN	50	F	634162	LABOUR	+	_	DIA	TN	DLSO	+	T.MENT	+	0.03	0.06	0.06	0.5			
141	Balamurugan	46	M	414612	LABOUR	+	_	_	FN	DLSO	_	_								
142	Ramamurthy	55	M	714614	LABOUR	_	_	IS	FN	PSO	+	TRICHOSPORON						0.5	0.5	0.03
143	Pasupaty	69	m	731421	LABOUR	+	_	dia	FN	TND	+	T.VERR	+	0.03	0.06	0.06	0.5			
144	Raja	68	M	701461	LABOUR	-	-	-	FN	DLSO	+	_	_							
145	NESAN	60	M	731461	FARMER	_	+	IS	TN	SWO	+	FUSARIUM						0.5	0.06	0.06
146	Siva	70	M	701412	LABOUR	+	_	DIA	F/TN	DLSO	+	T.VERR	+	0.03	0.06	0.06	1			
147	Nargeez Banu	41	F	731612	MISC	-	N	_	TN	TND	+	SYNCEPHALASTRUM						0.06	0.03	0.03
148	Abdul Jaffer	33	M	731412	LABOUR	+	_	DIA	FN	DLSO	_	_	+							
149	Jahira Banu	50	F	724142	HOUSEWIFE	~	~	DIA	FN	PSO	+	C.ALBICANS					0.06	0.03	ket - 0.03	0.03
150	Chithran	34	M	731415	LABOUR	neg	~	~	FN	`~	_	-	_							

KEY TO MASTER CHART

Male - M
Female - F
No growth - NG
Distal Lateral Subungual Onychomycosis - DLSO
Proximal Lateral Subungual Onychomycosis - PSO
Total dystrophic Onychomycosis - TDO
Superficial white Onychomycosis - SWO
Minimum inhibitory concentration - MIC
50 % of Isolates inhibited - MIC_{50}
90% of Isolates inhibited - MIC_{90}
Labourers - LAB
Students - STU
Farmers - FAR
Diabetes - DIA
Immunosuppression - IS
Finger nail - FN
Toe nail - TN

PROFORMA

NAME;
AGE/SEX;
IP/OP NUMBER;
OCCUPATION;
ADDRESS;
COMPLAINTS;

HISTORY OF PRESENT ILLNESS
ONSET;
DURATION;
PROGRESS;
H/O PAIN;
H/O TRAUMA –Y/N
H/O USE OF SHOES-Y/N
H/O EXCESSIVE SWEATING-Y/N
H/O SIMILAR ILLNESS INVOLVING SKIN OR HAIR –Y/N
H/O PRIMARY DERMATOSES INVOLVING NAIL –Y/N

PAST HISTORY;
H/O SIMILAR ILLNESS IN THE PAST
H/O DIABETES
H/O MALIGNANCY
H/O DRUG INTAKE;
PERSONAL HISTORY ;
H/O SMOKING;
FAMILY HISTORY;
TRATMENT HISTORY;
CLINICAL FEATURES;
TREATMENT HISTORY;
GENERAL EXAMINATION;
LOCAL EXAMINATION;
SITE
COLOUR –YELLOWISH OR BROWNISH STREAKS
SURFACE –THICKENING /RIDGING
SUBUNGUAL HYPERKERATOSIS –Y/N
ONYCHOLYSIS –Y/N
DIAGNOSIS –ONYCHOMYCOSIS –DLSO/PSO/TND/SWO

INVESTIGATIONS;
Blood sugar
HIV Elisa for
KOH (DMSO)-
CULTURE-
SDA WITH ANTIBIOTICS AND CYCLOHEXIMIDE-
SDA WITH ANTIBIOTICS-
POLYMERASE CHAIN REACTION-
ANTIFUNGAL SUSCEPTIBILITY METHOD-
E TEST METHOD-

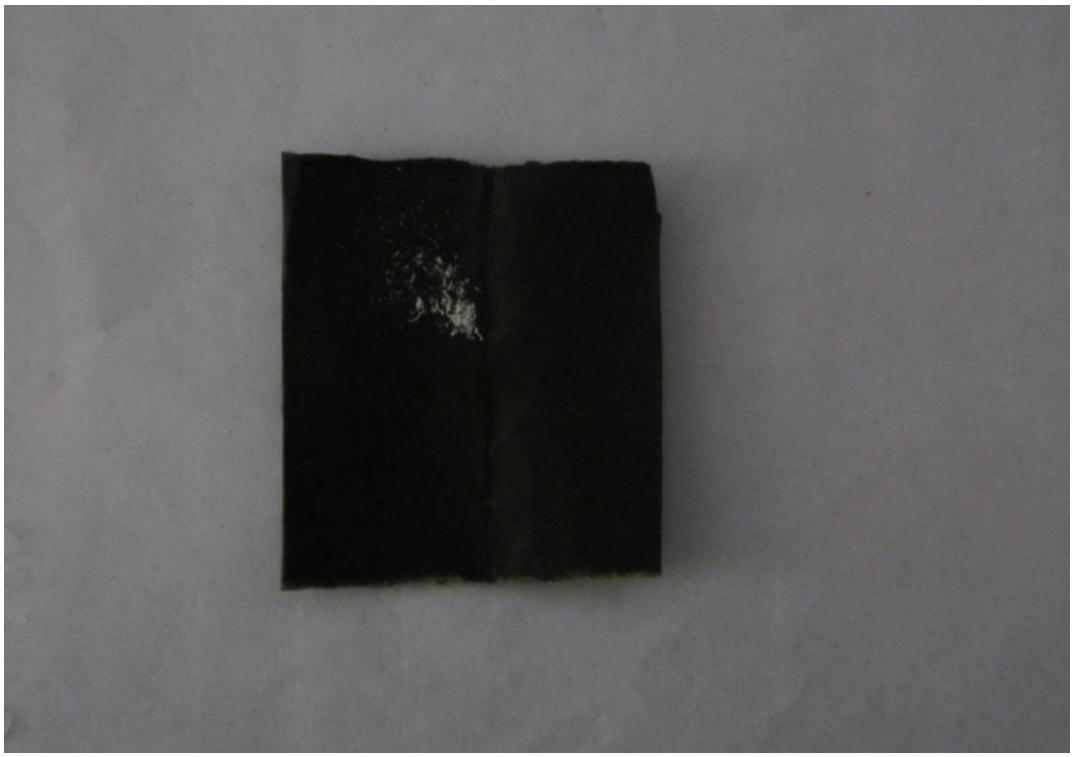
DLSO

PSO

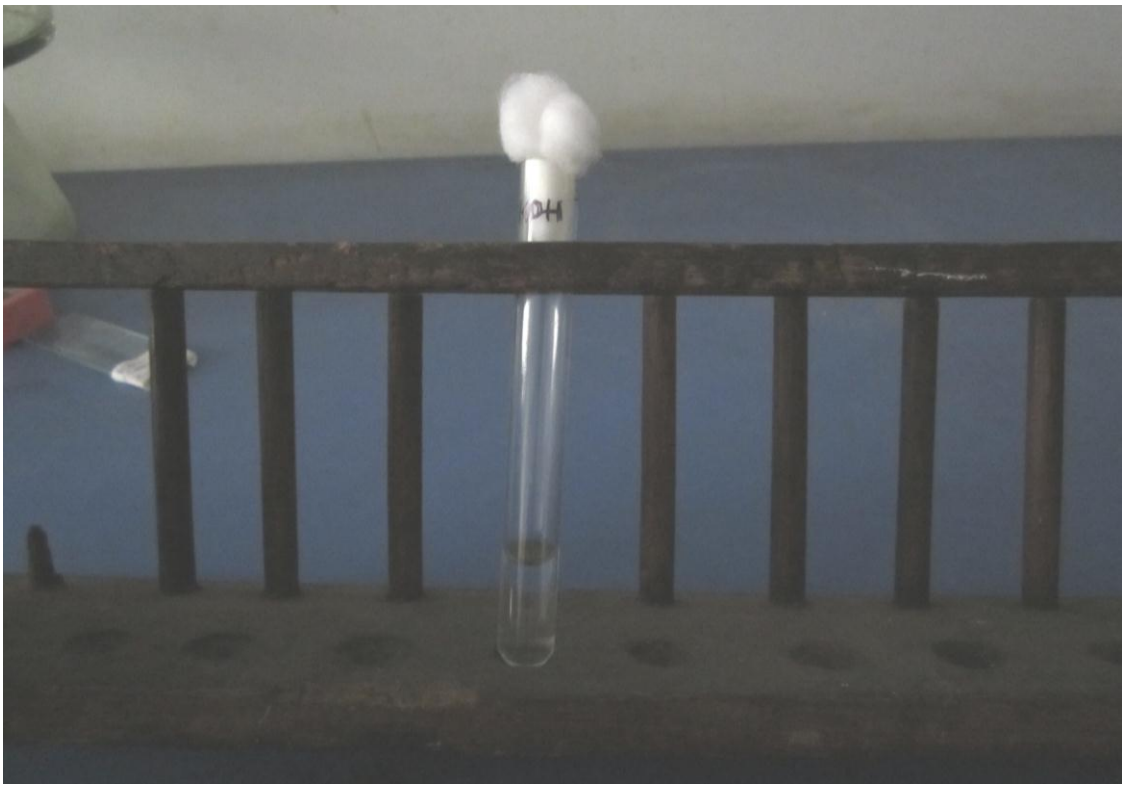
SWO

TND

SPECIMEN COLLECTION KIT



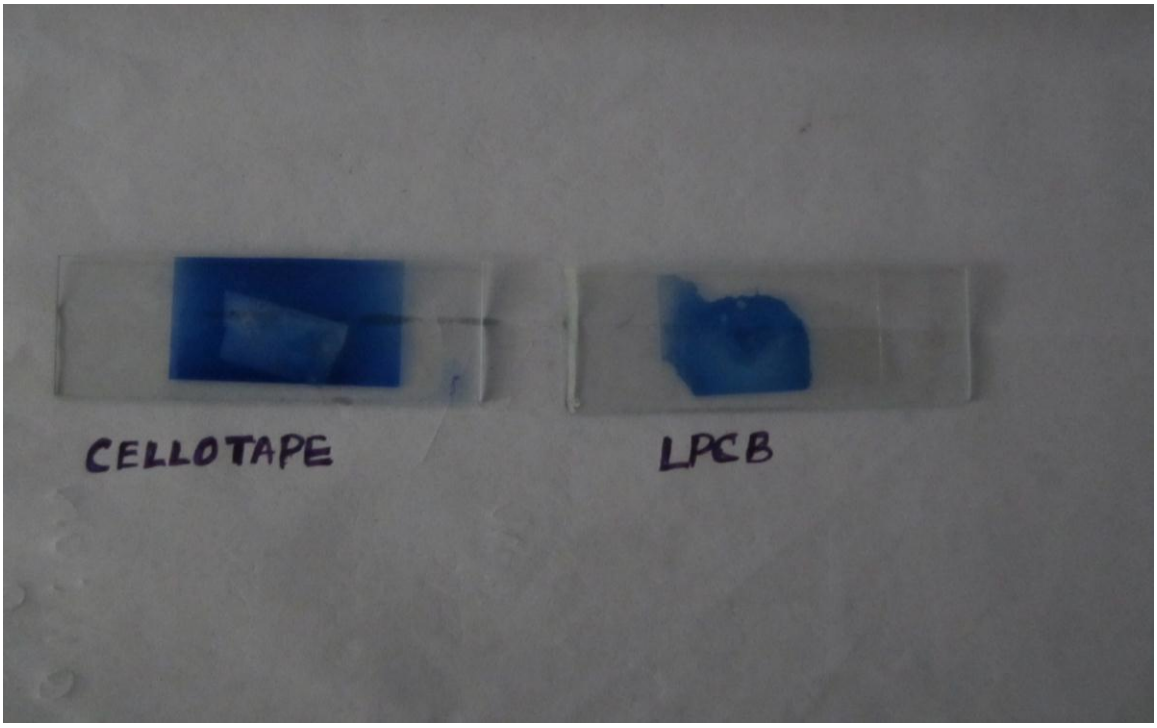
STERILE KRAFT PAPER



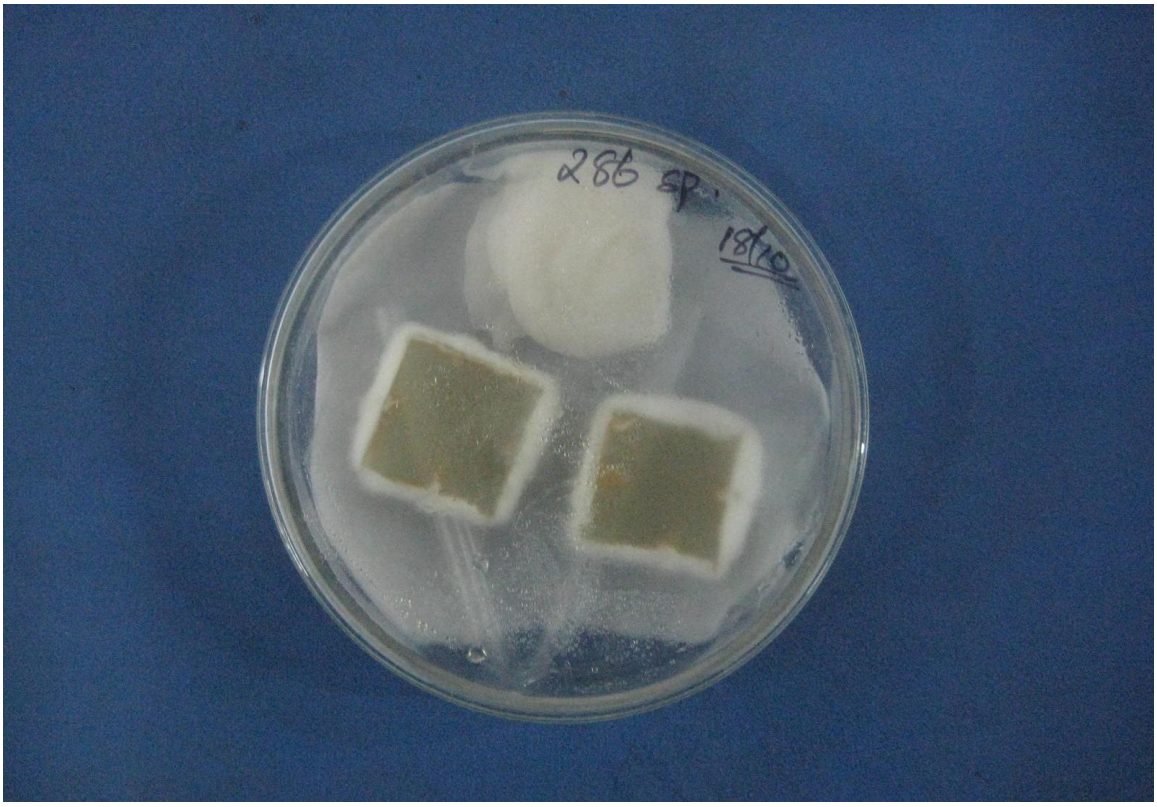
KOH WITH DMSO

PROCESSING KIT

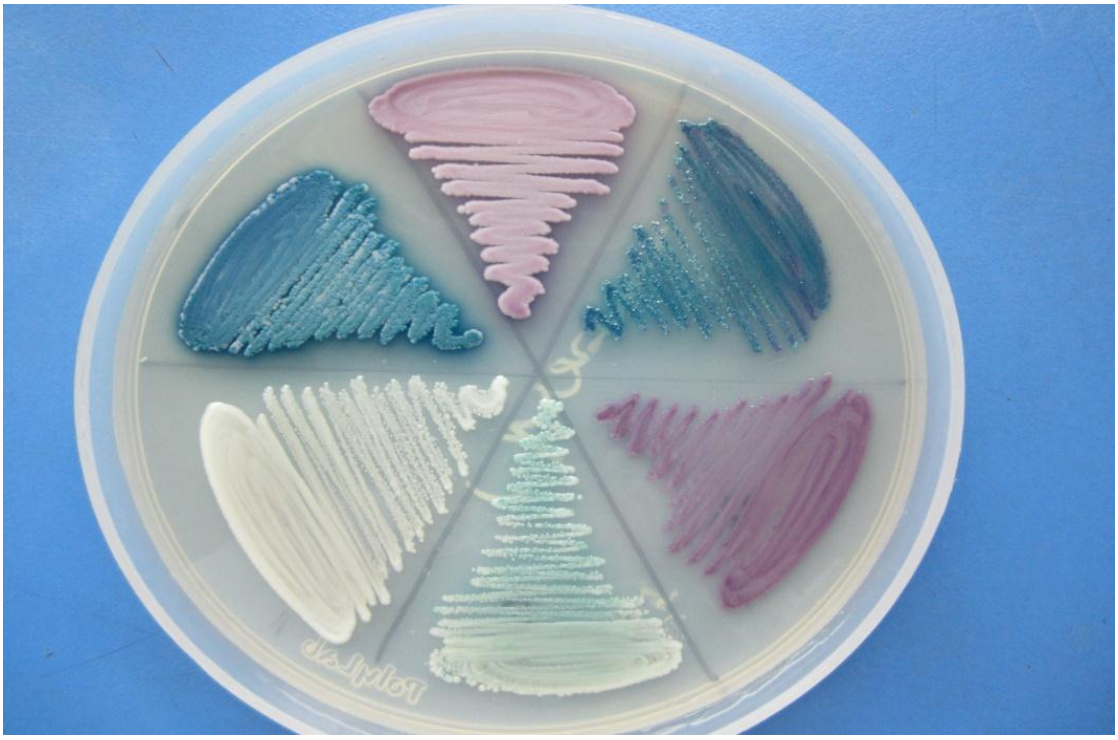
SDA SLANT



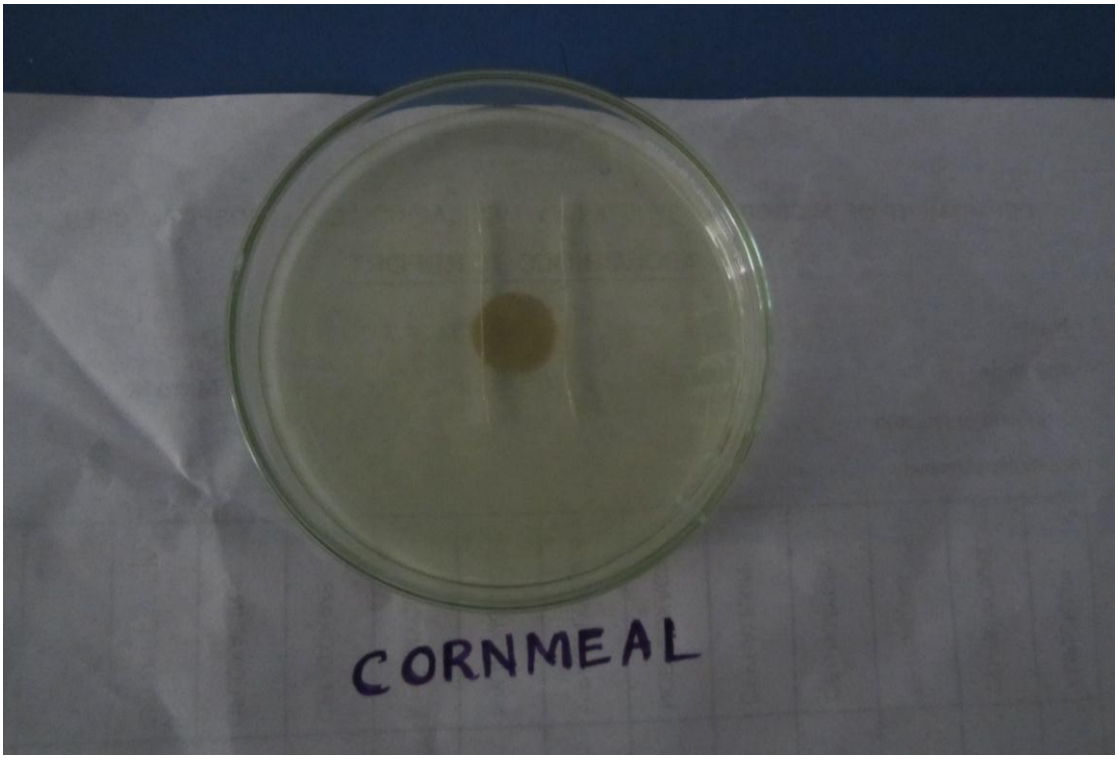
TEASE MOUNT AND CELLOPHANE



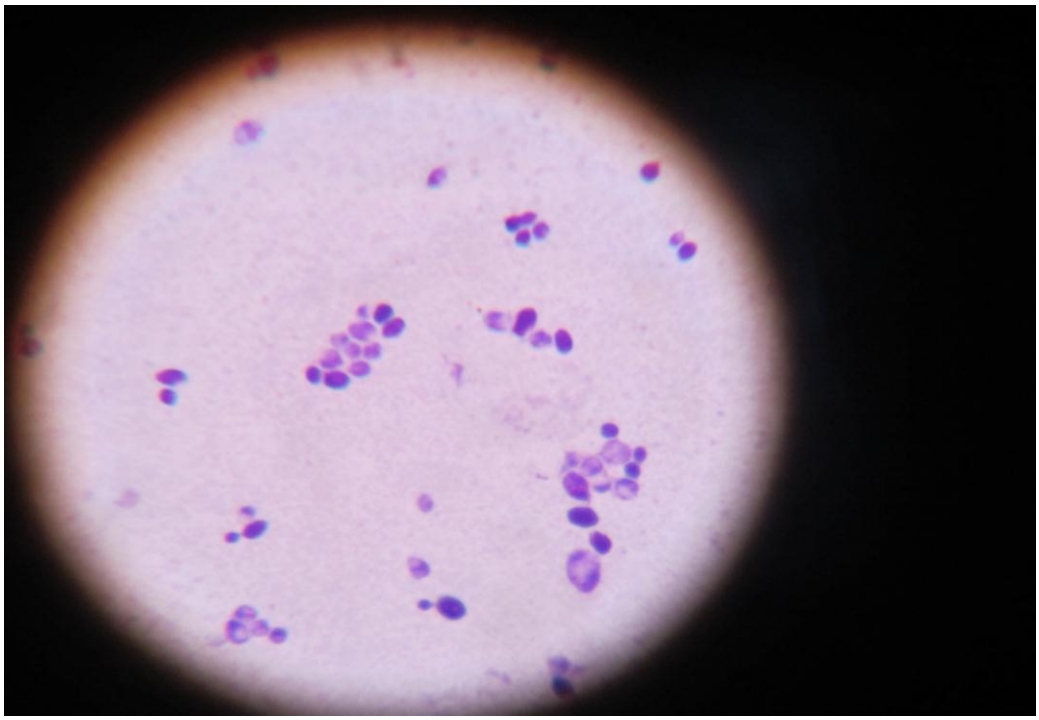
SLIDE CULTURE



CHROME AGAR

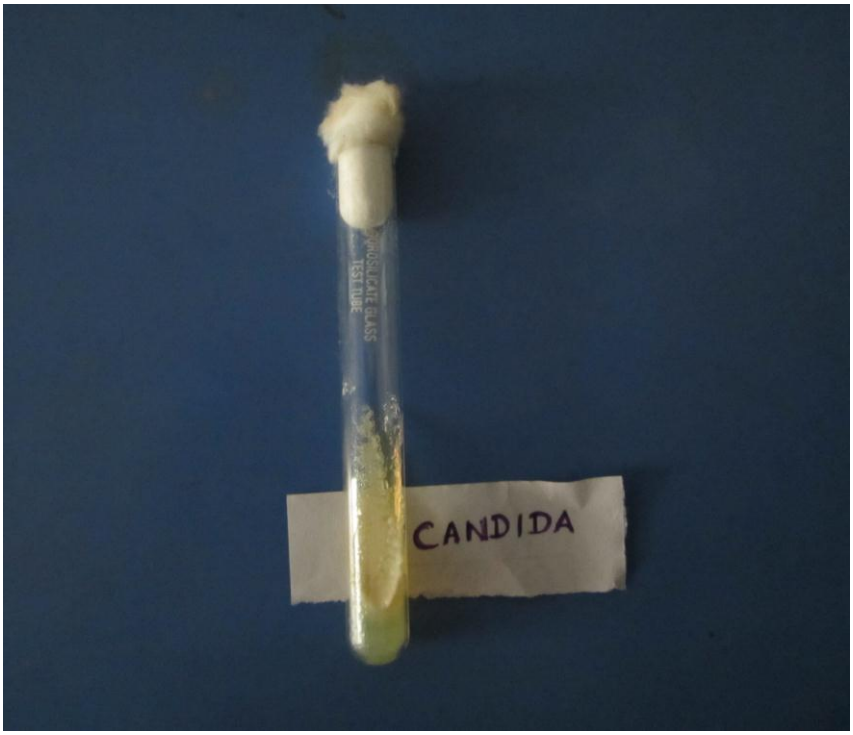


CORNMEAL AGAR

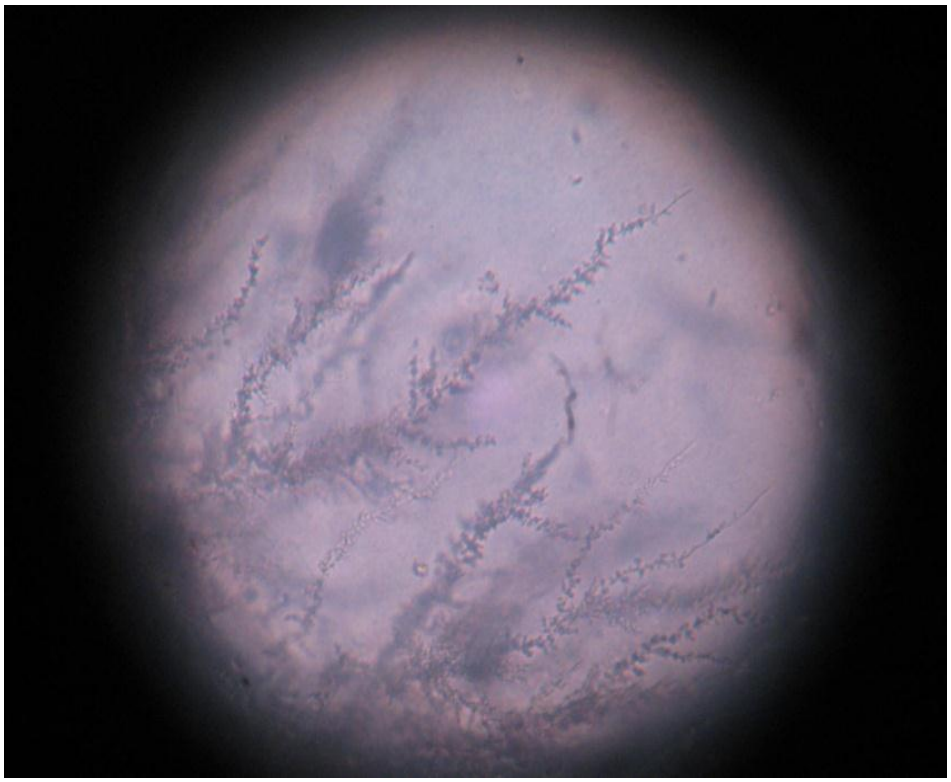


GRAM STAIN – CANDIDA

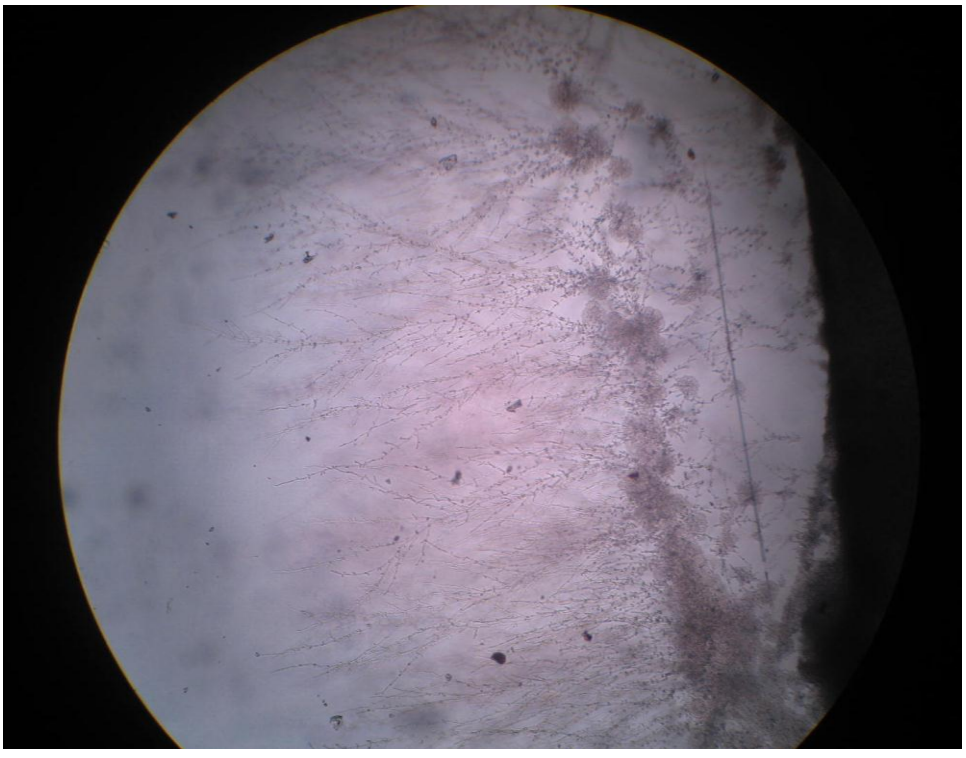
CANDIDA – GERM TUBE FORMATION



C. ALBICANS

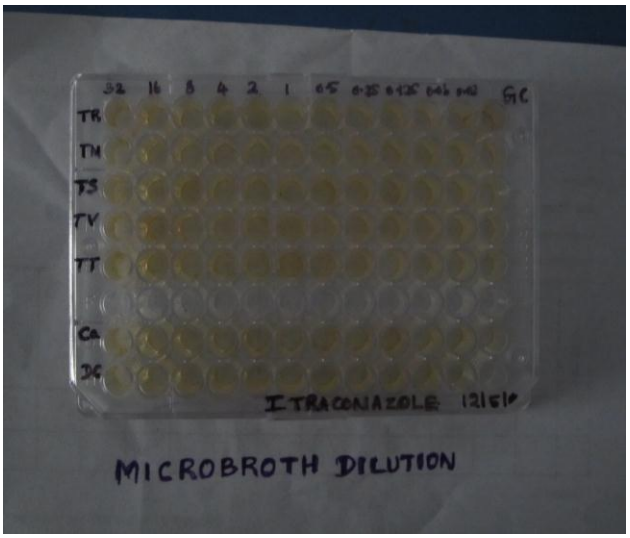


C. TROPICALIS



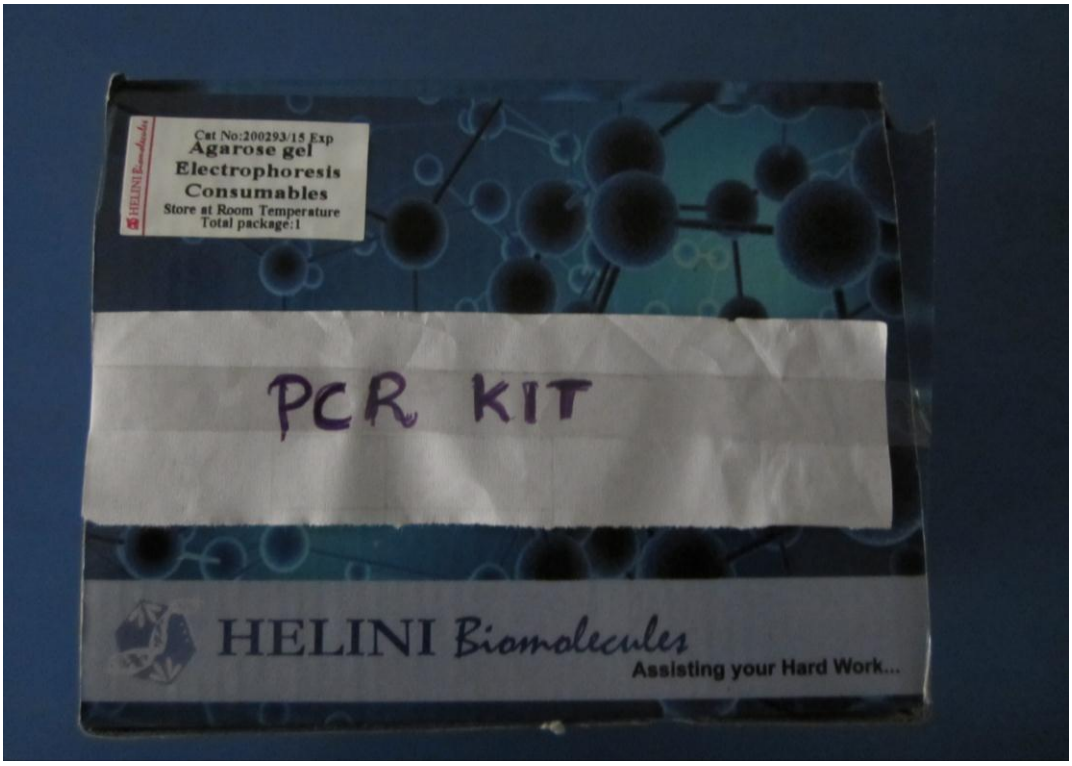
C. PARAPSILOSIS

SPECTRO PHOTOMETER

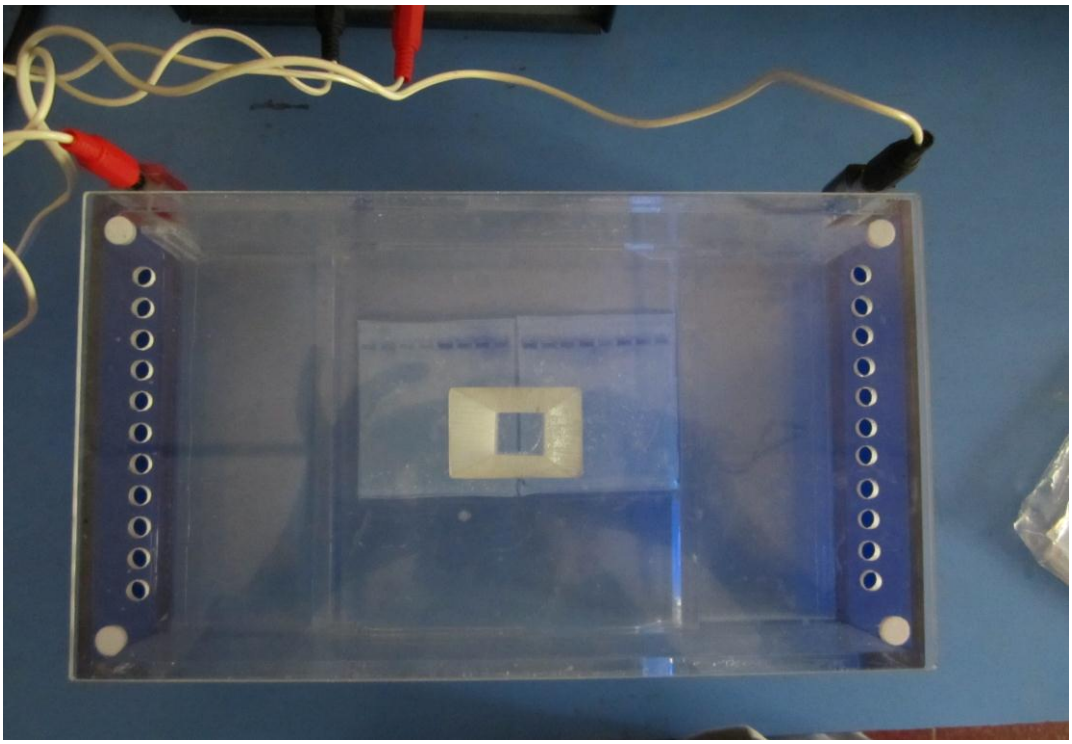


MICROBROTH DILUTION PLATE

DRUG DILUTION - STOCK



PCR KIT

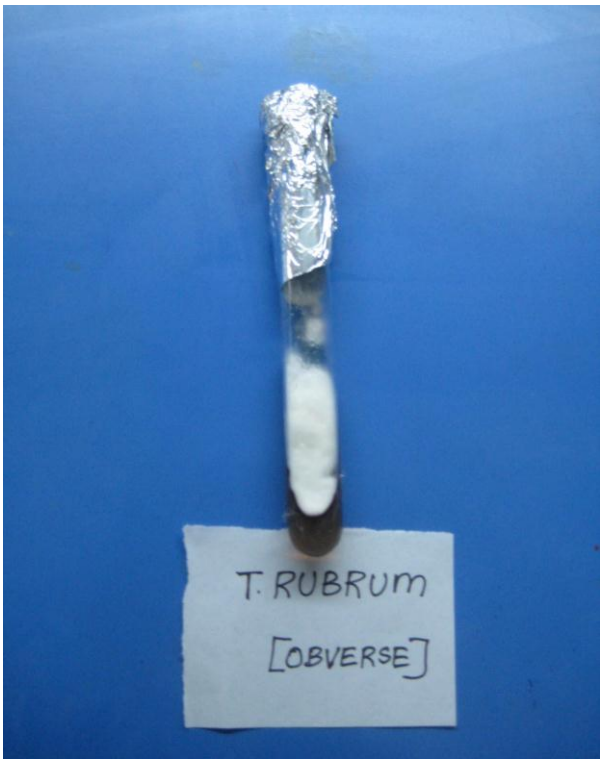


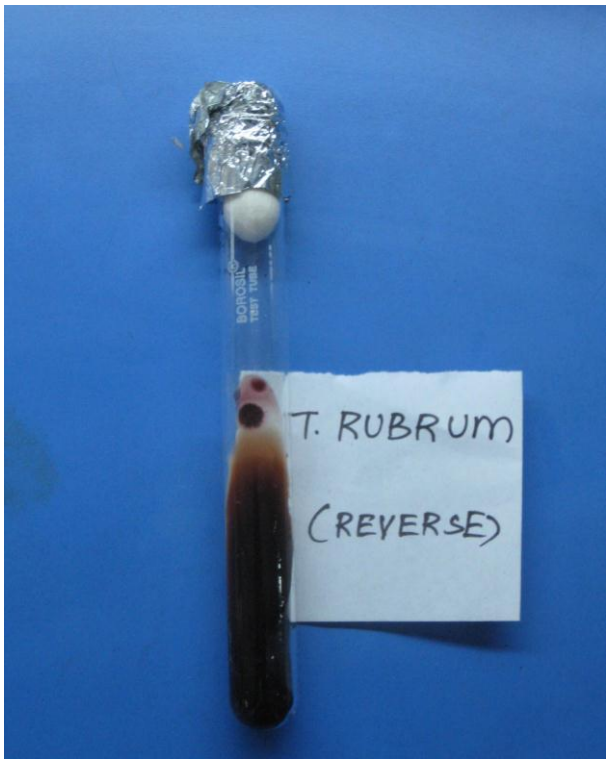
ELECTROPHORESIS UNIT

THERMOCYCLER

UV TRANSILLUMINATOR

T.RUBRUM





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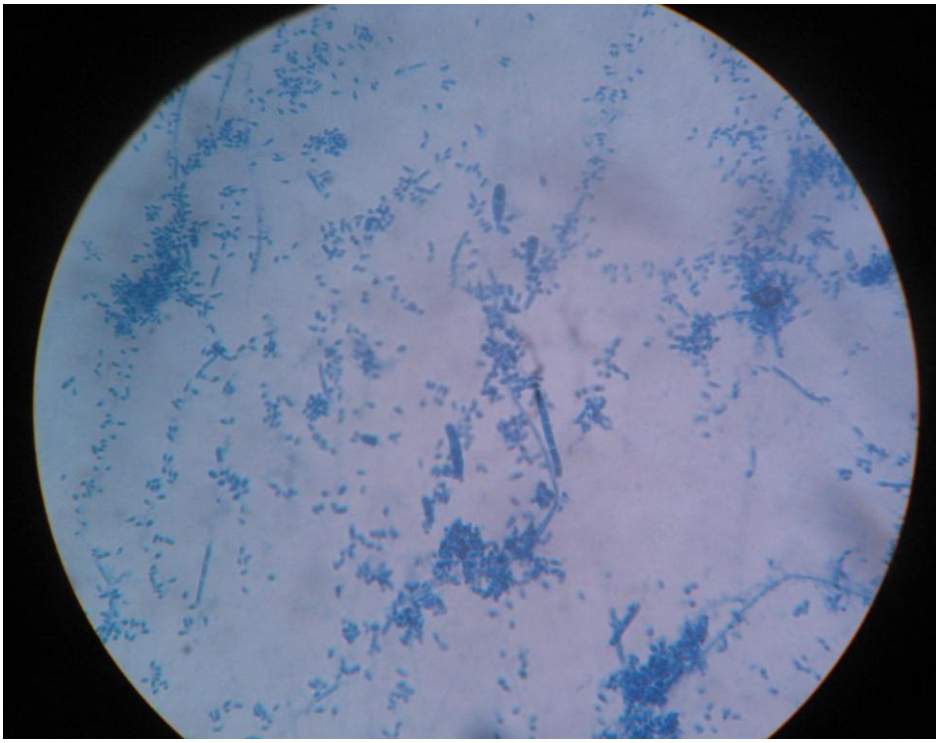
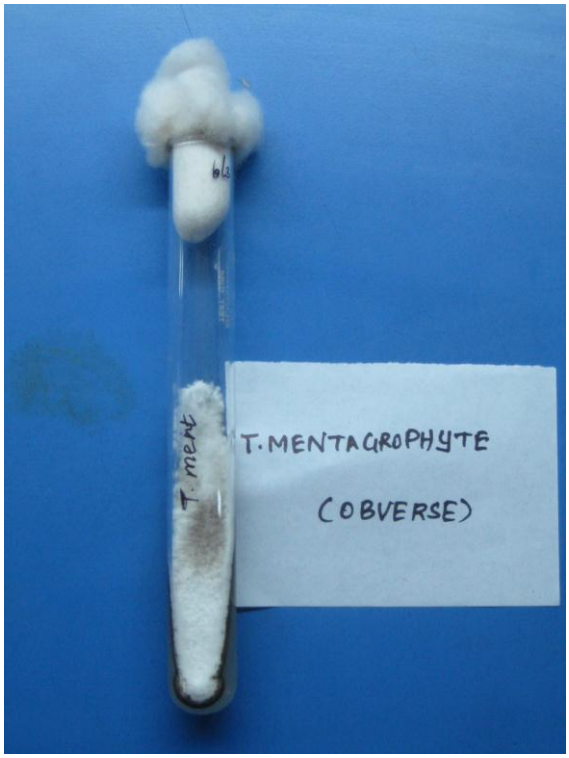


MICROSCOPY

T.MENTAGROPHYTES



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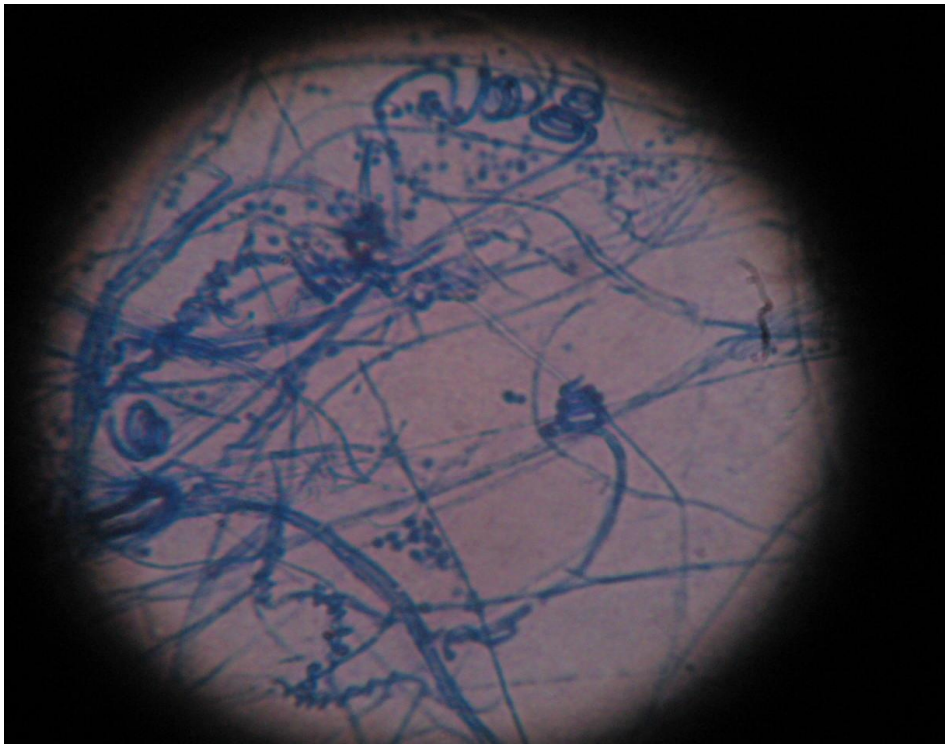

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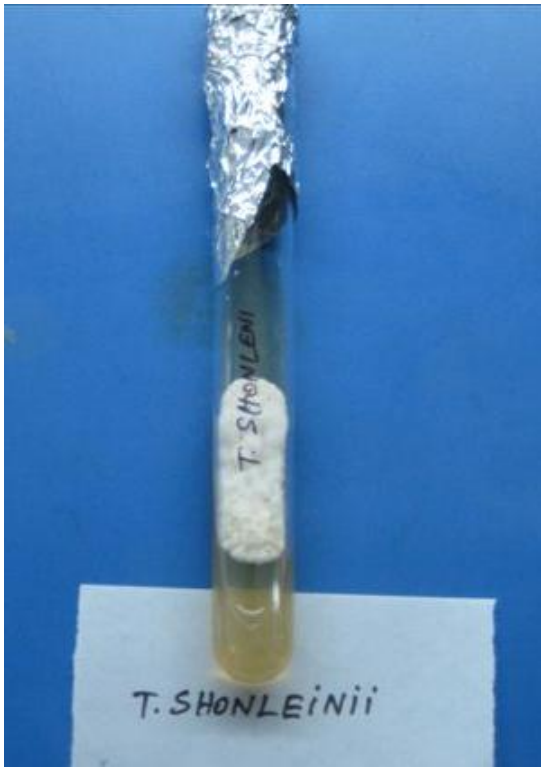
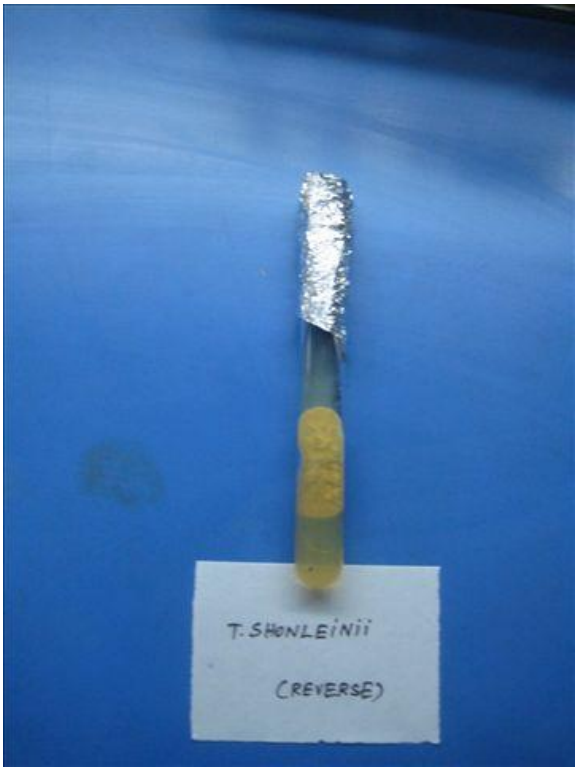
T.SHONLEINII





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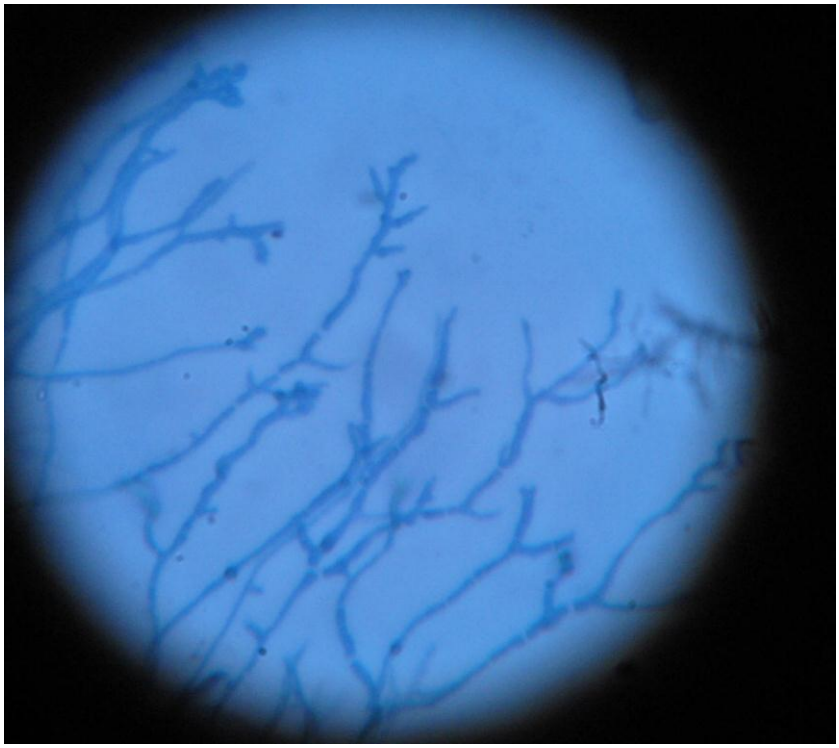
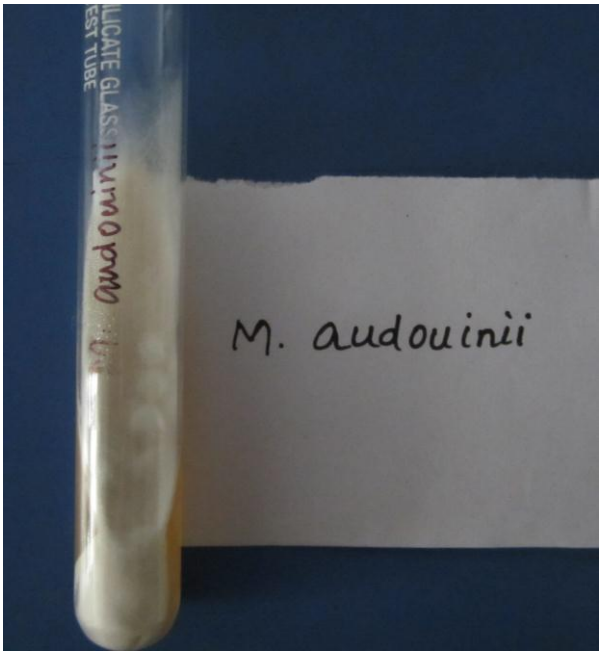
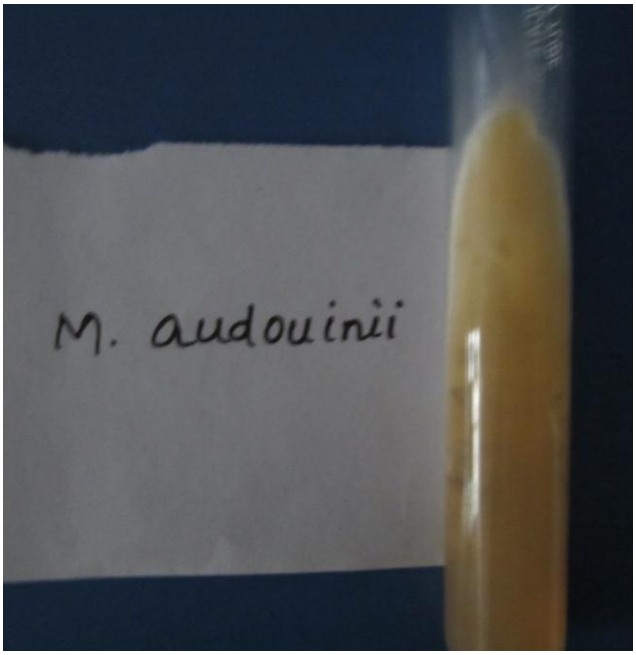


MICROSCOPY

M.AUDOUINII





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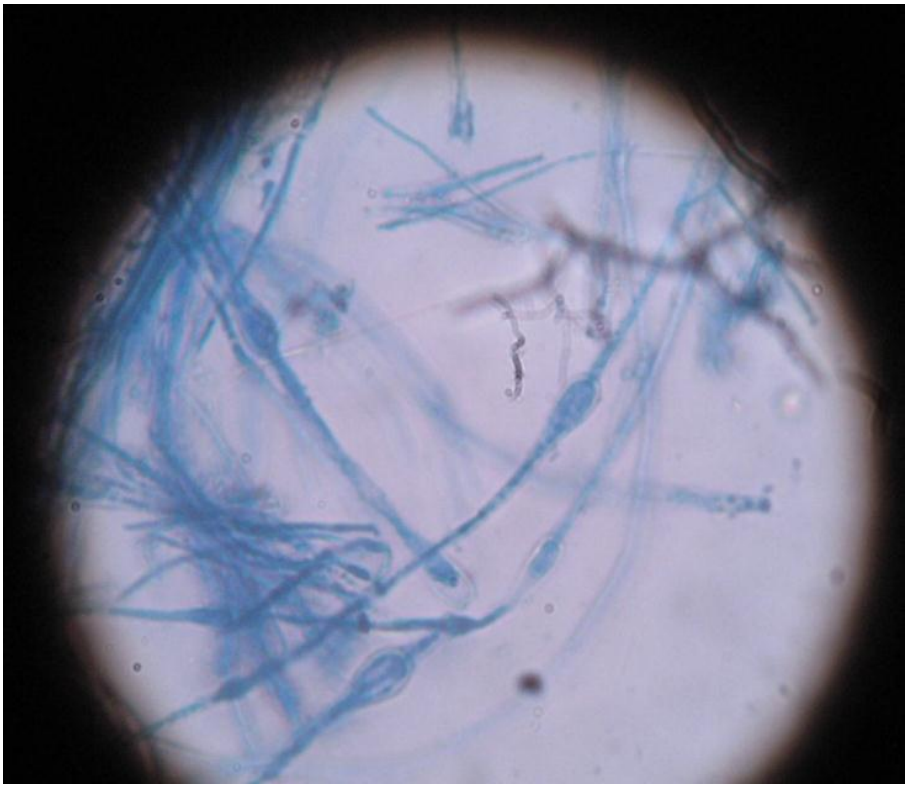
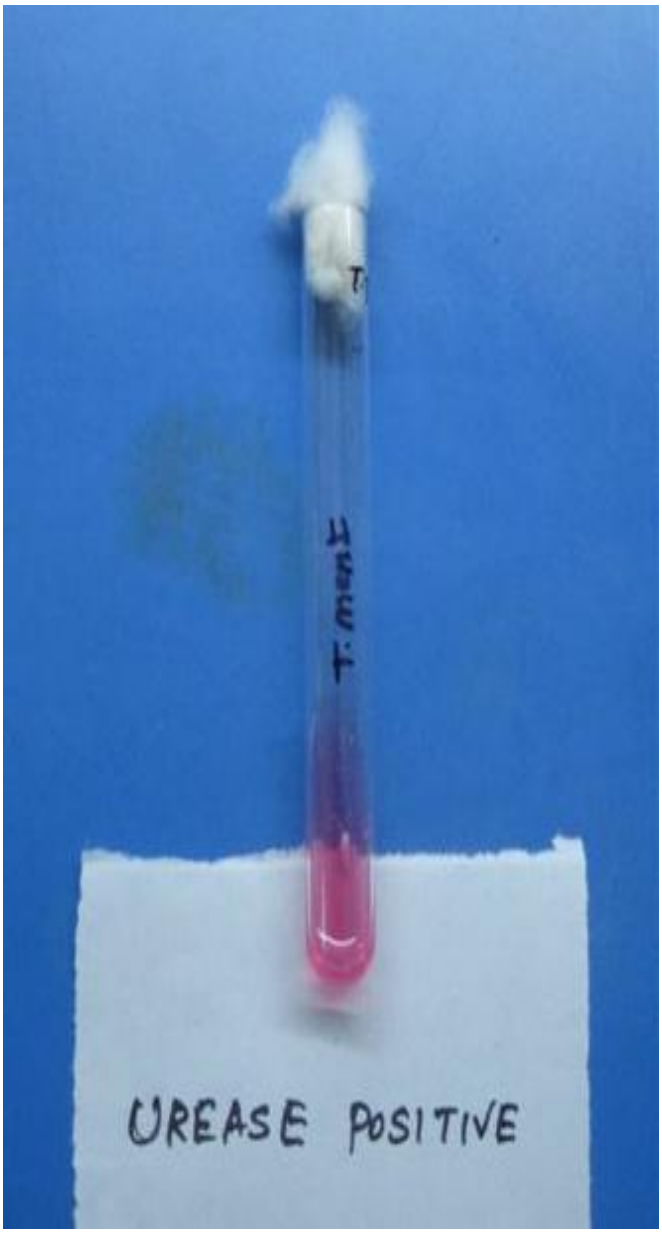
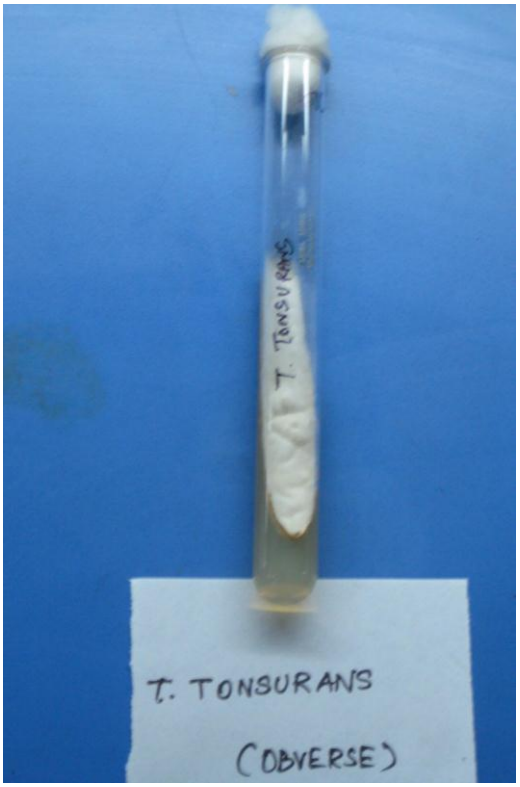


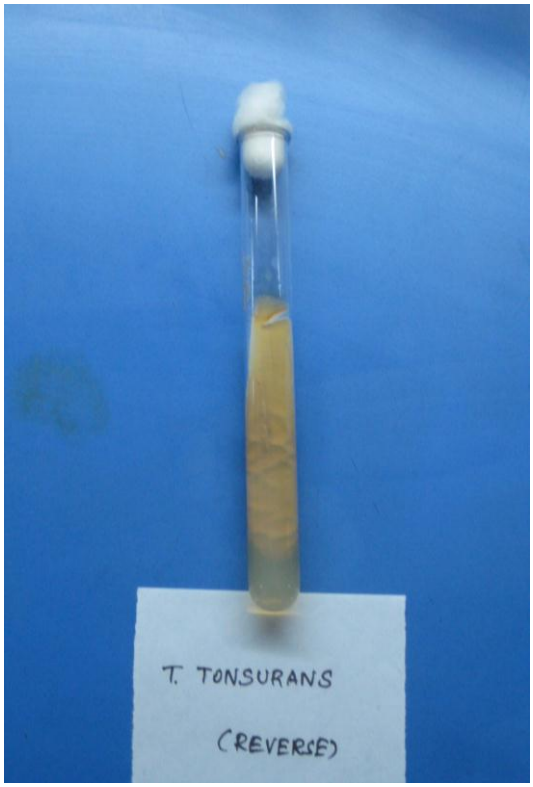
MICROSCOPY

CHRISTENSEN'S UREASE AGAR



T. TONSURANS





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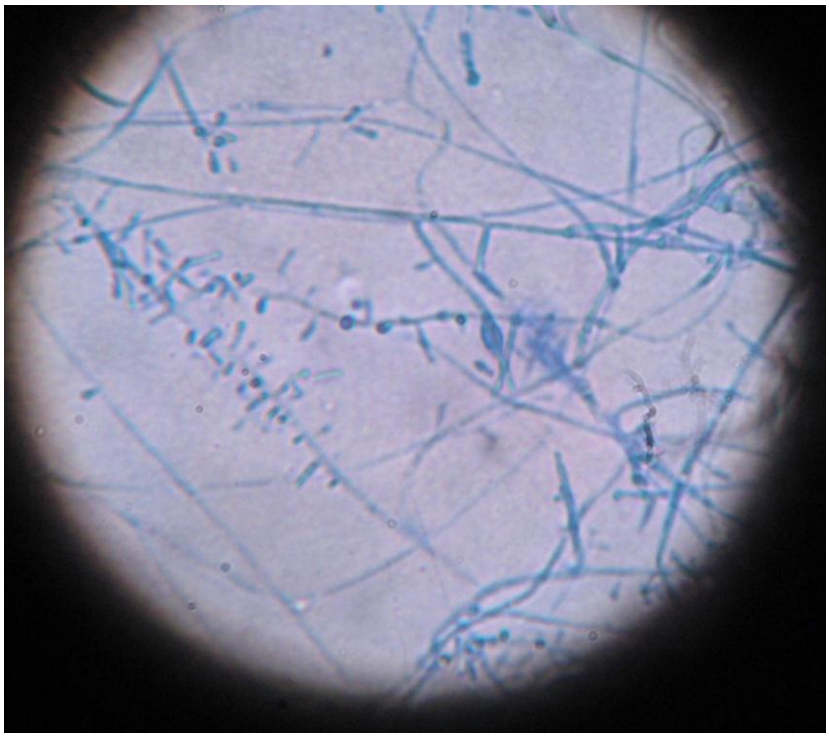
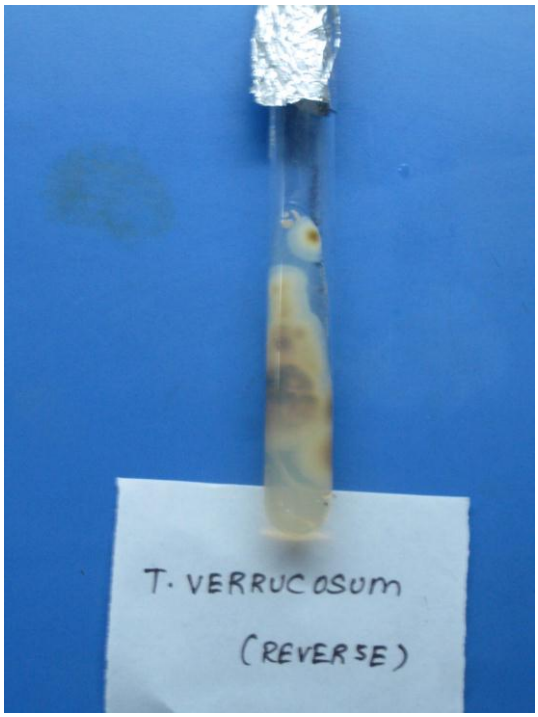


MICROSCOPY

T.VERRUCOSUM

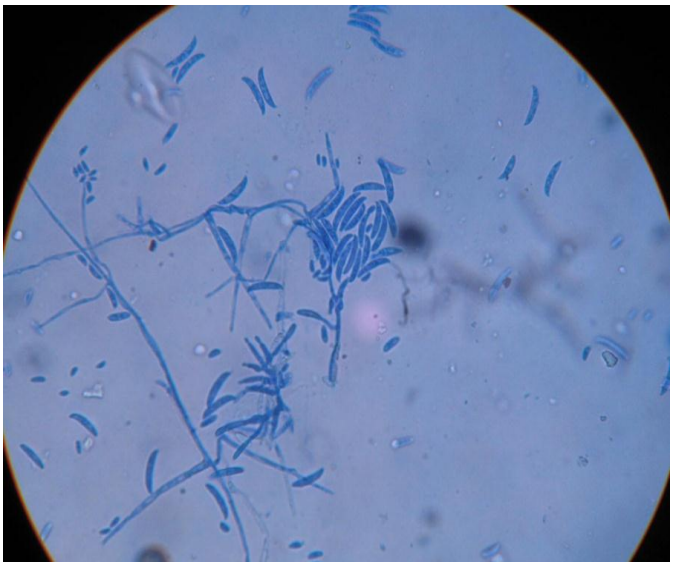


MACROSCOPY

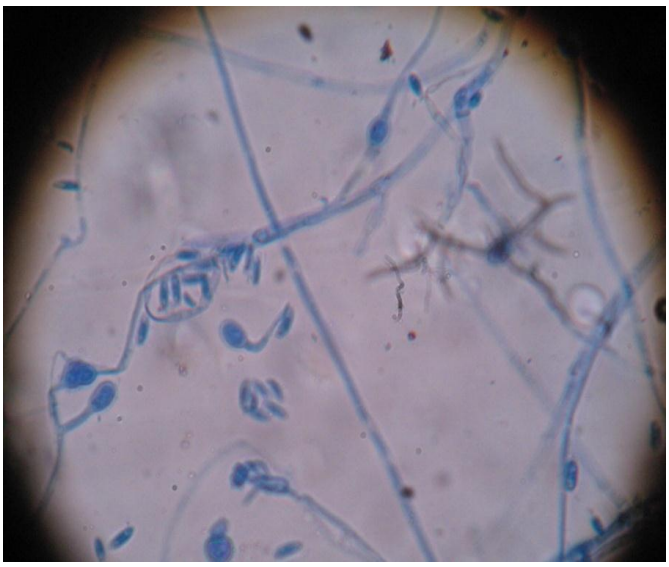
MICROSCOPY

FUSARIUM

MACROSCOPY



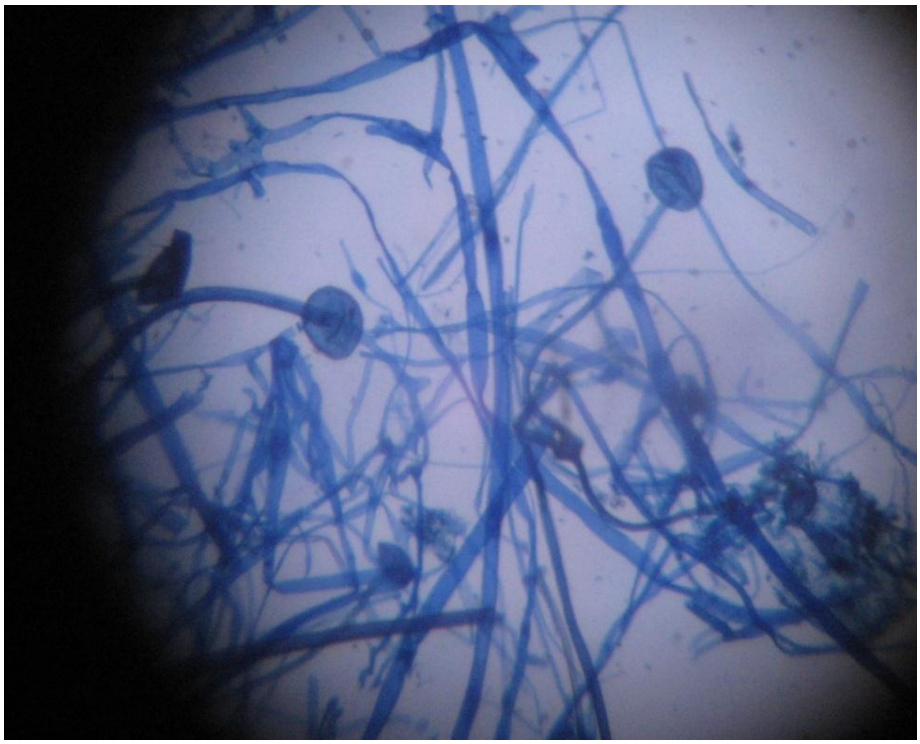
F.OXYSPORUM



F. SOLANI

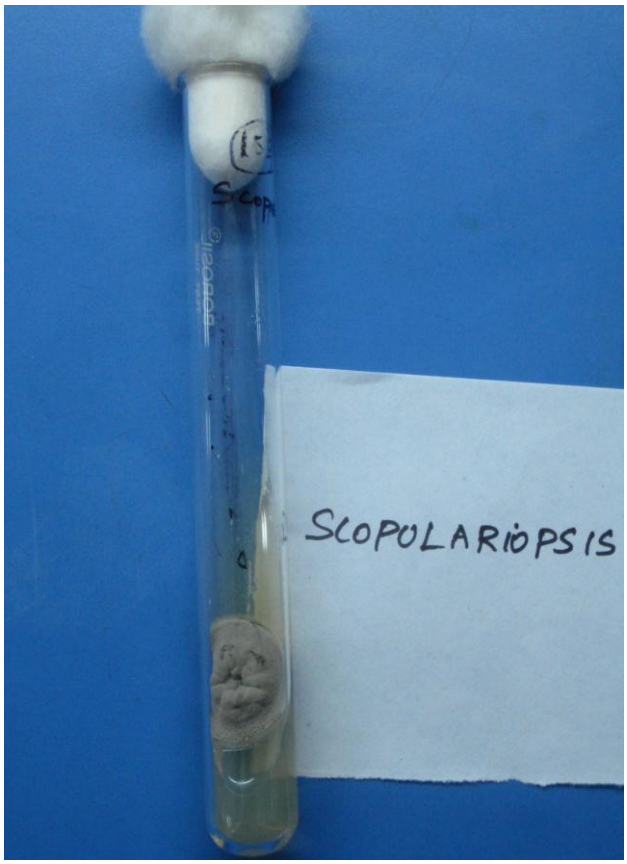
RHIZOPUS

MACROSCOPY



MICROSCOPY

SCOPULARIOPSIS



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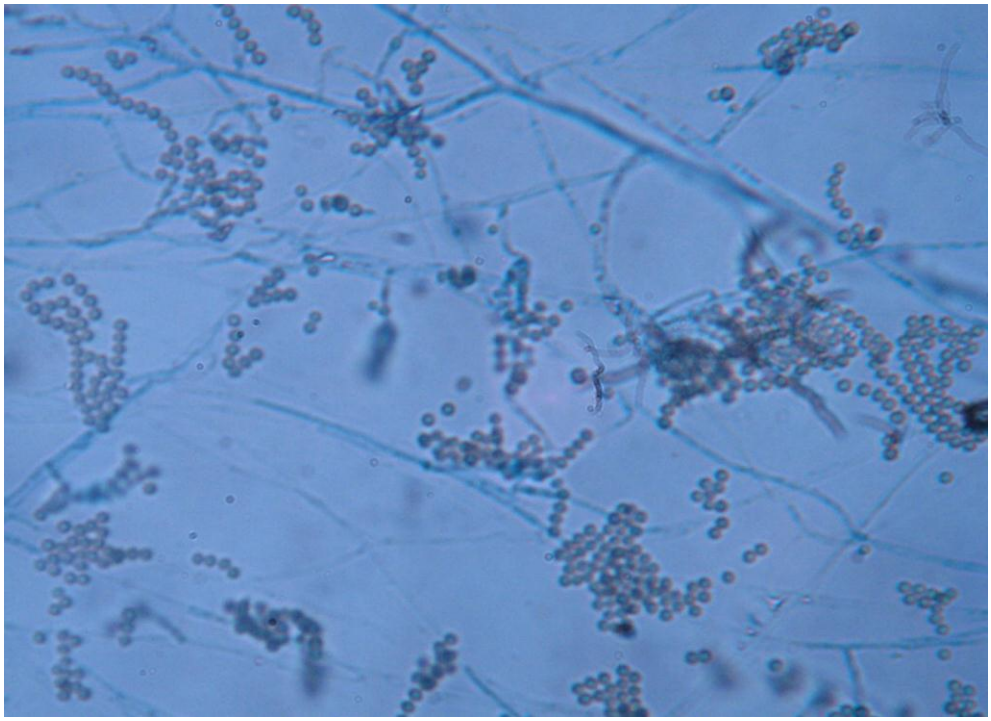
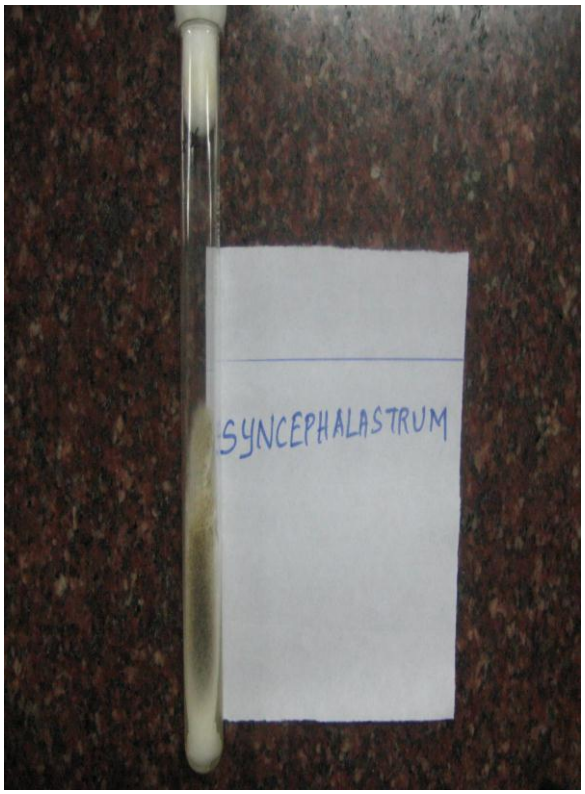


MICROSCOPY

SYNCEPHALASTRUM



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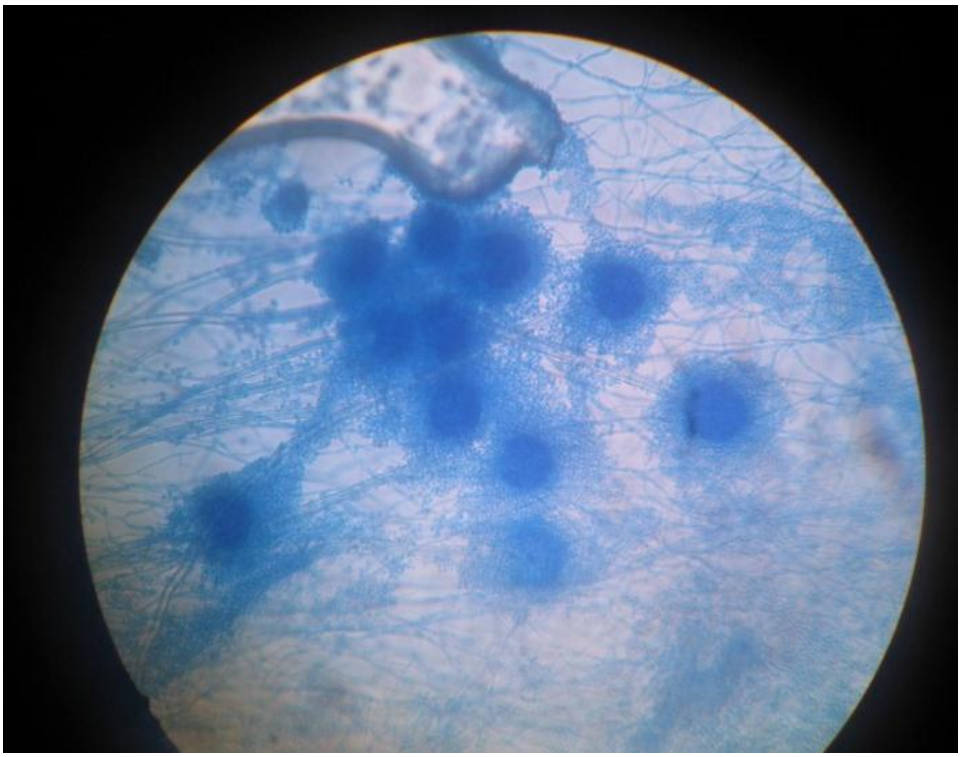
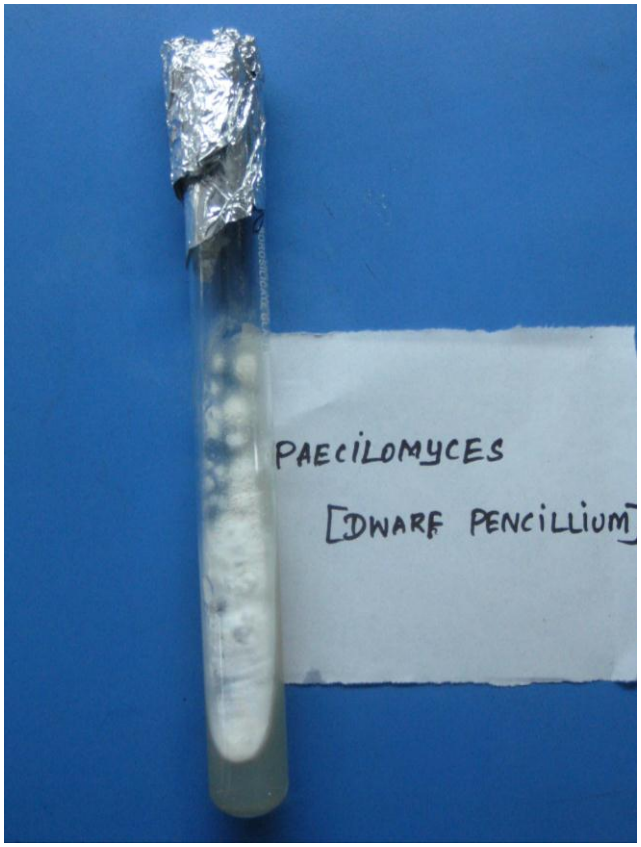


MICROSCOPY

PAECILOMYCES



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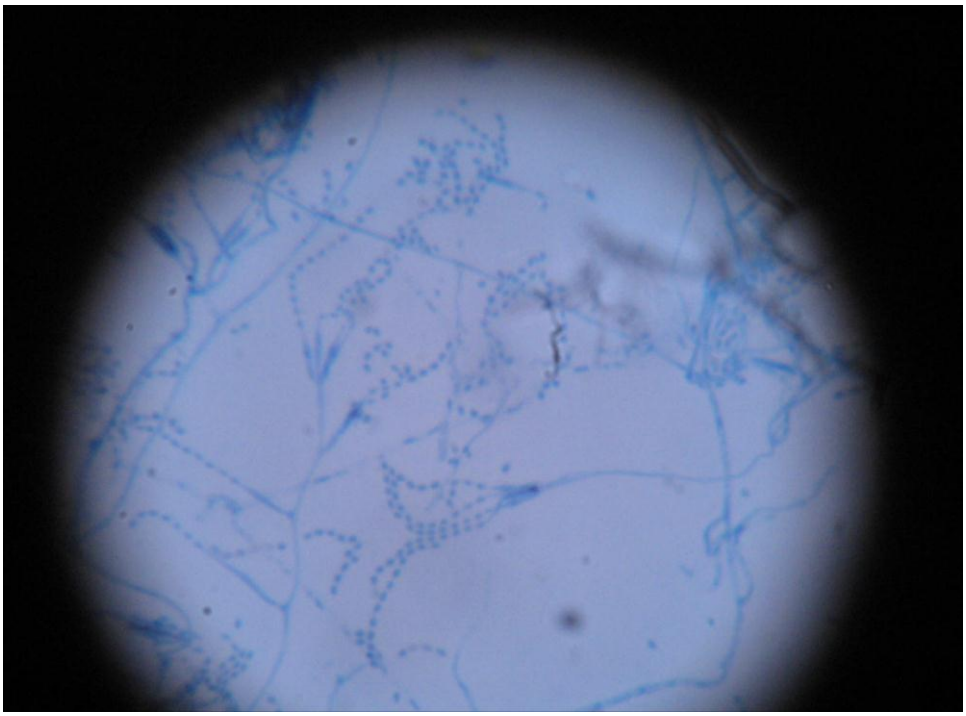
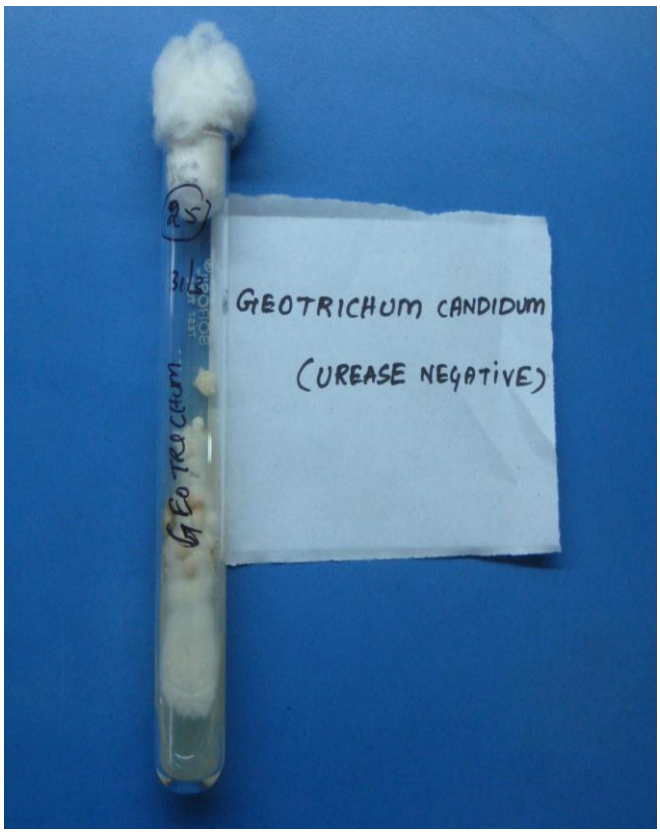


MICROSCOPY

GEOTRICHUM CANDIDUM



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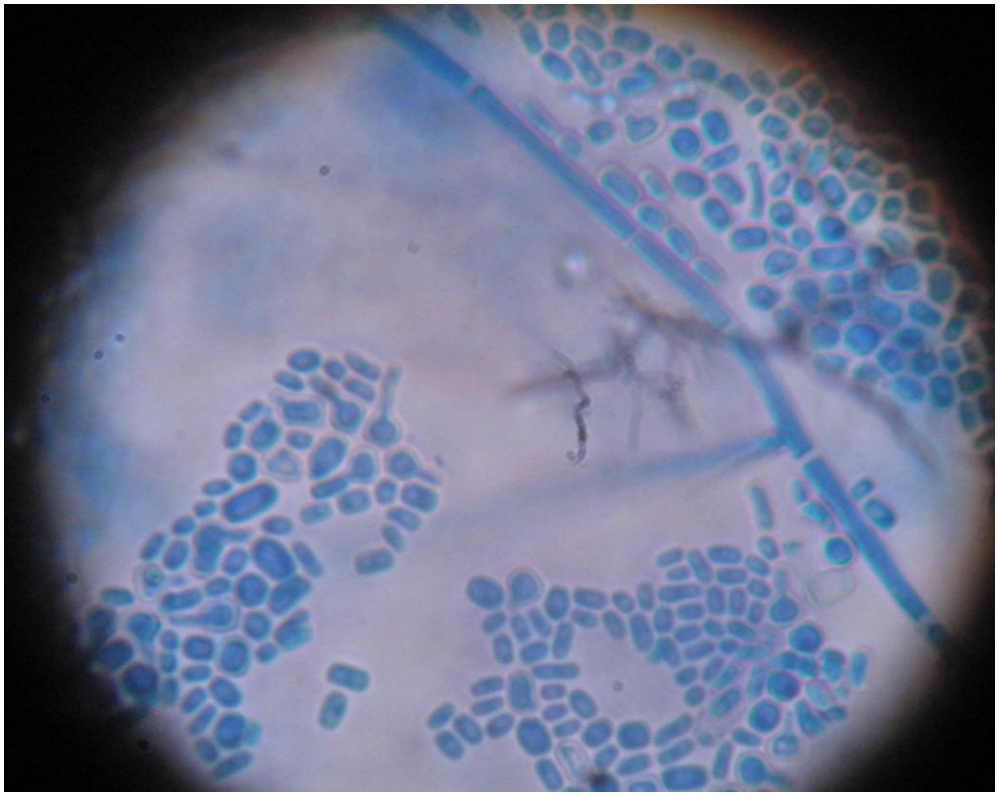
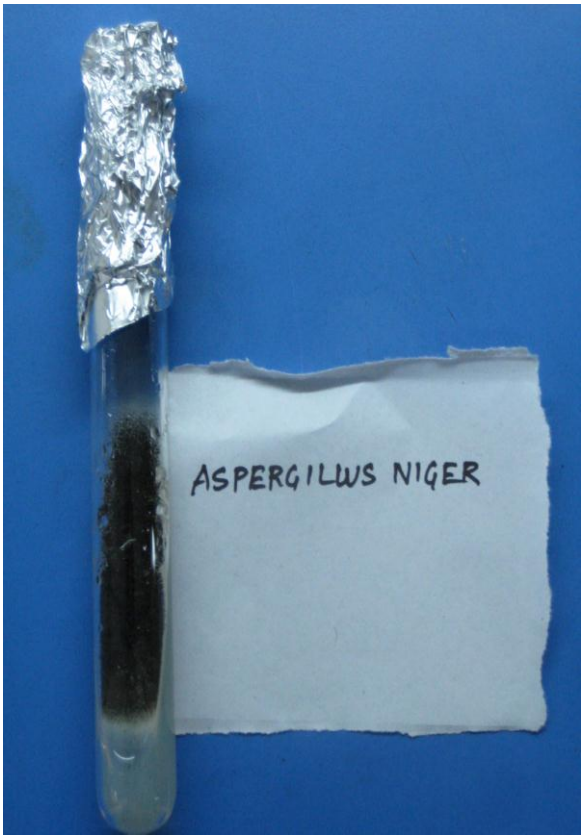
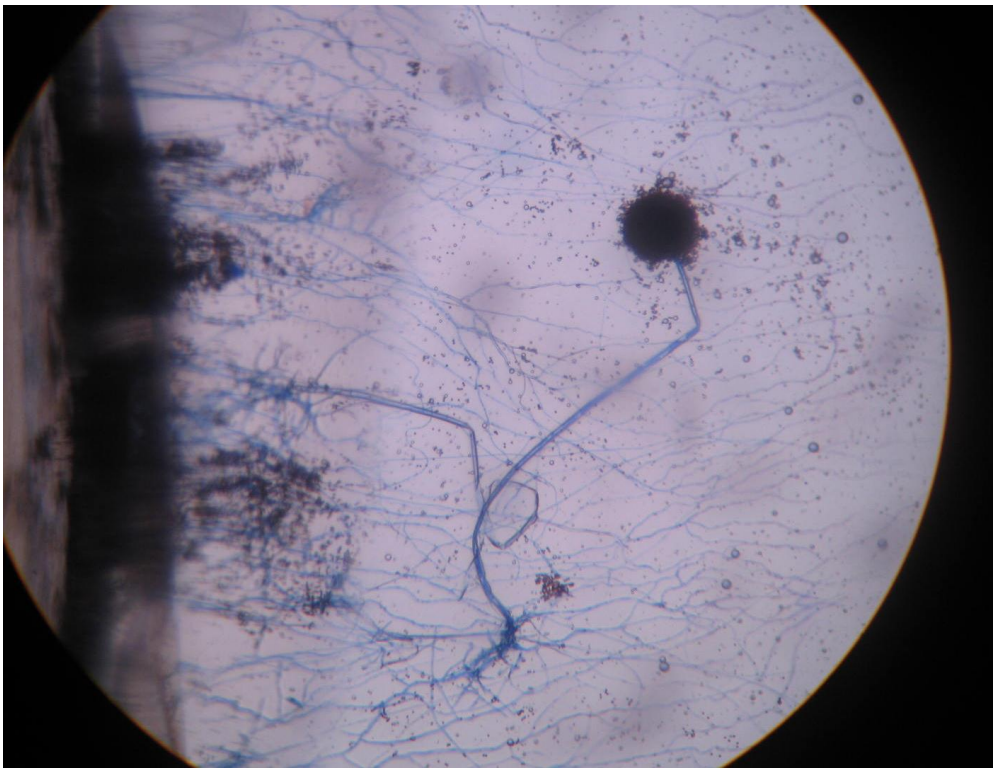


MICROSCOPY

A. NIGER



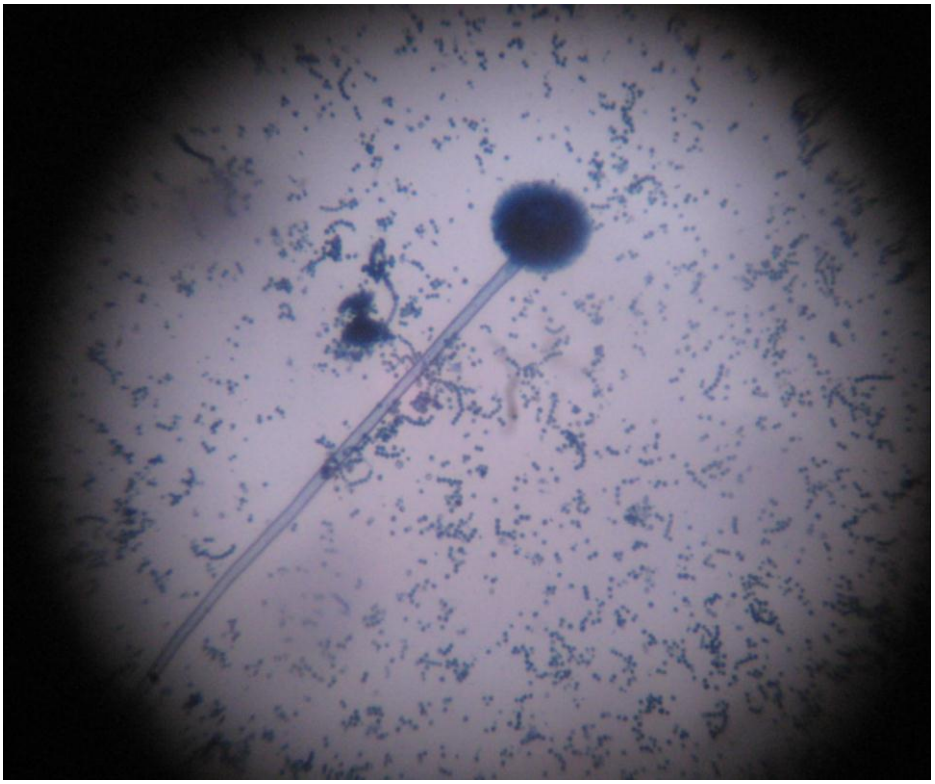
MACROSCOPY



MICROSCOPY

A.FLAVUS

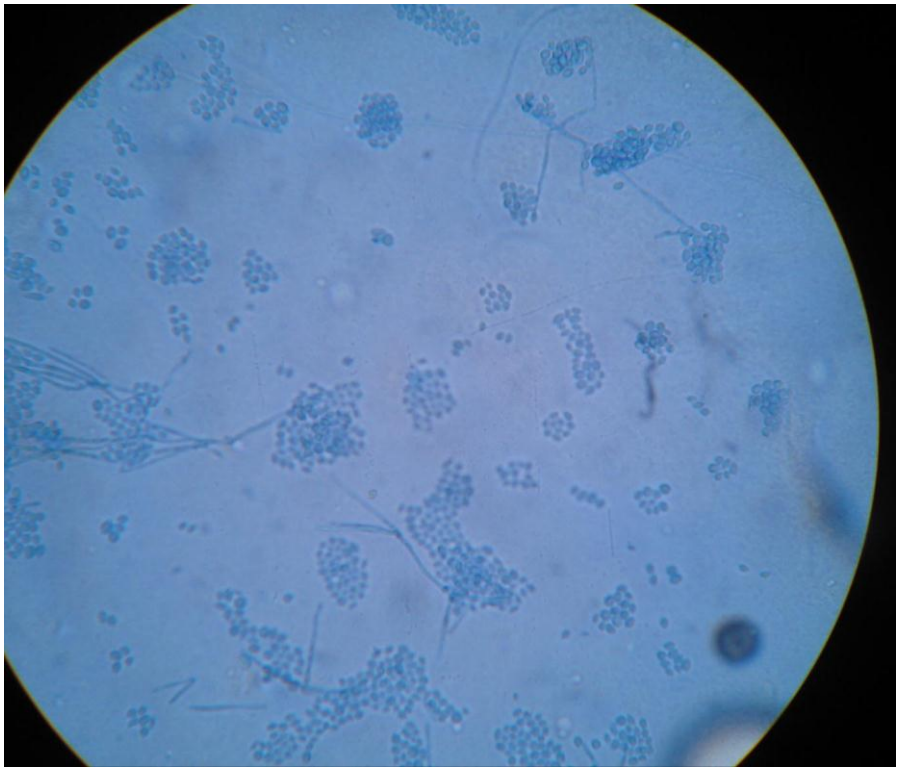
MACROSCOPY



MICROSCOPY

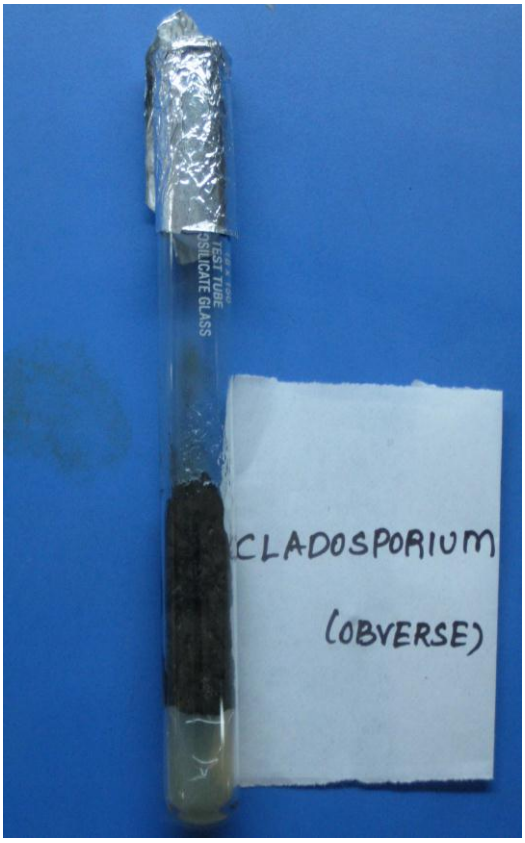
ACREMONIUM

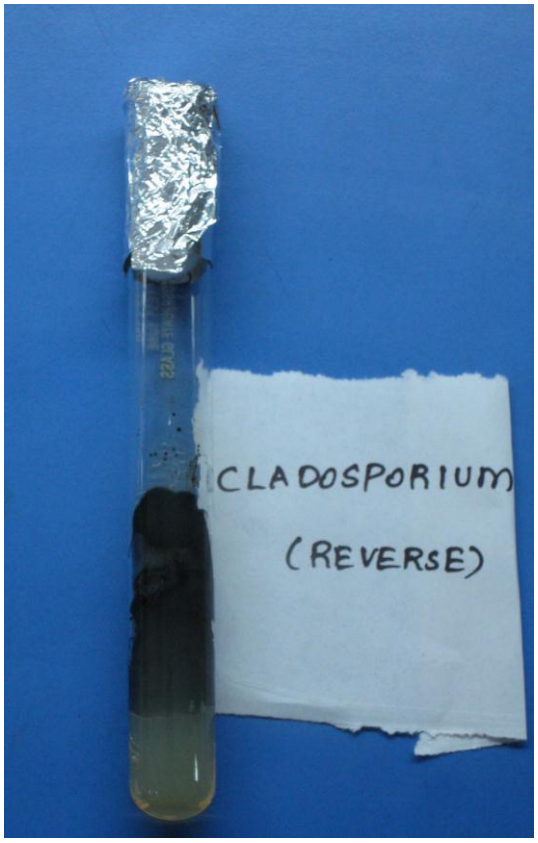
MACROSCOPY



MICROSCOPY

CLADOSPORIUM





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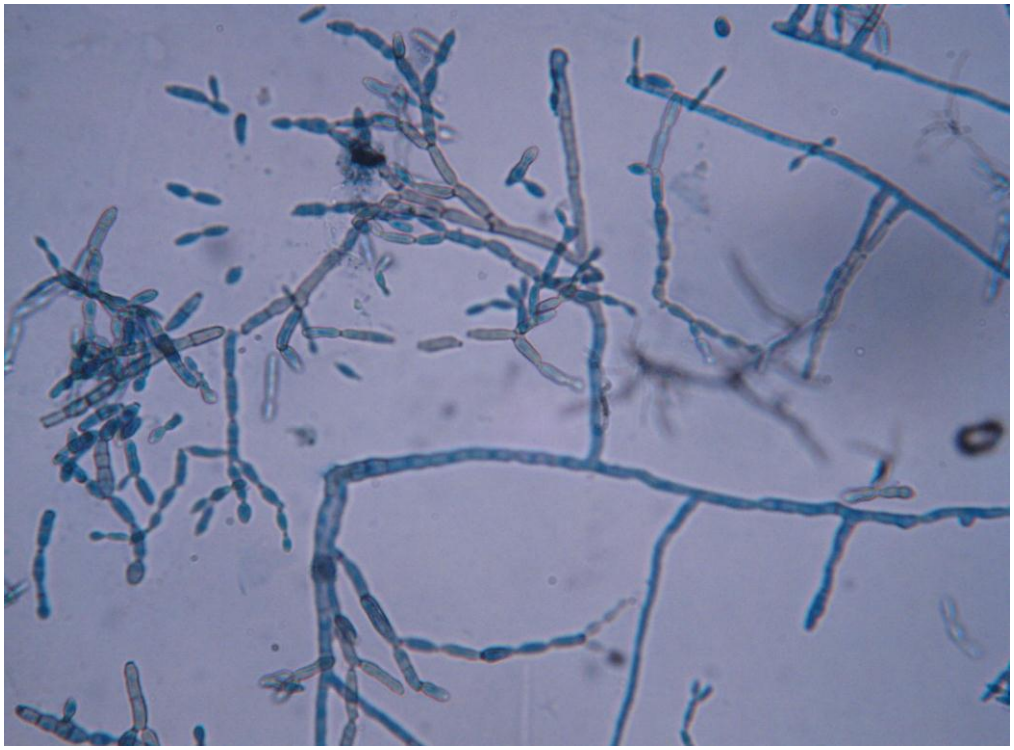
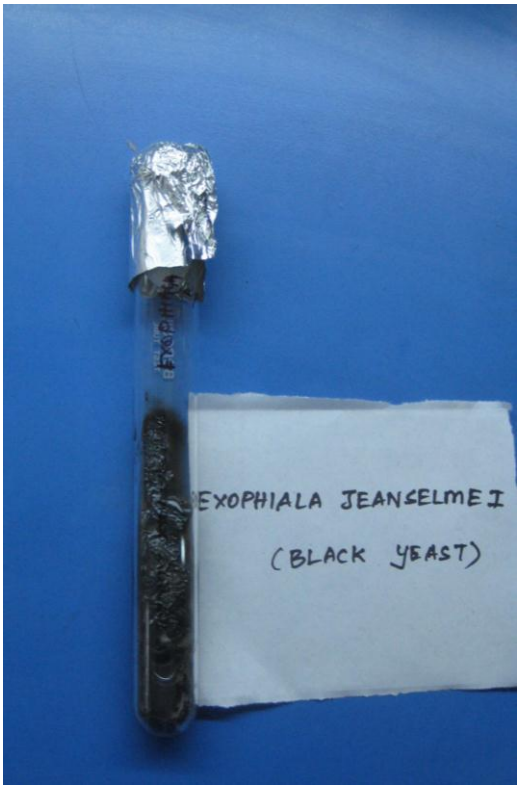
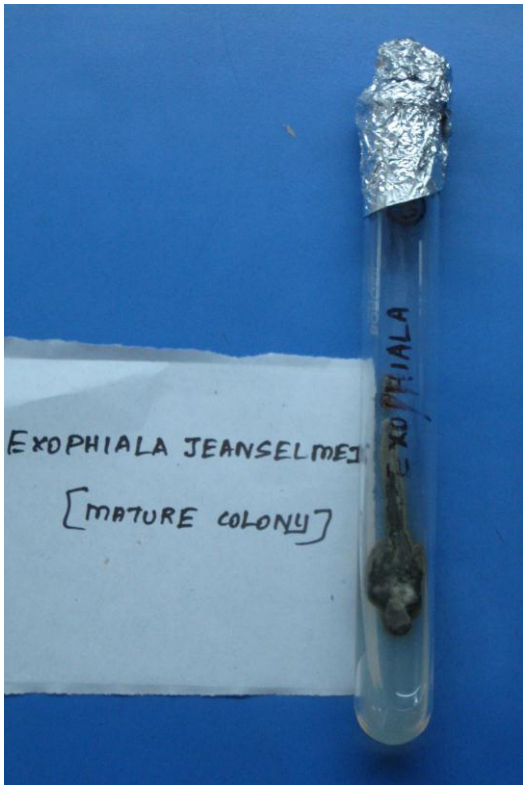


MICROSCOPY

EXOPHIALA





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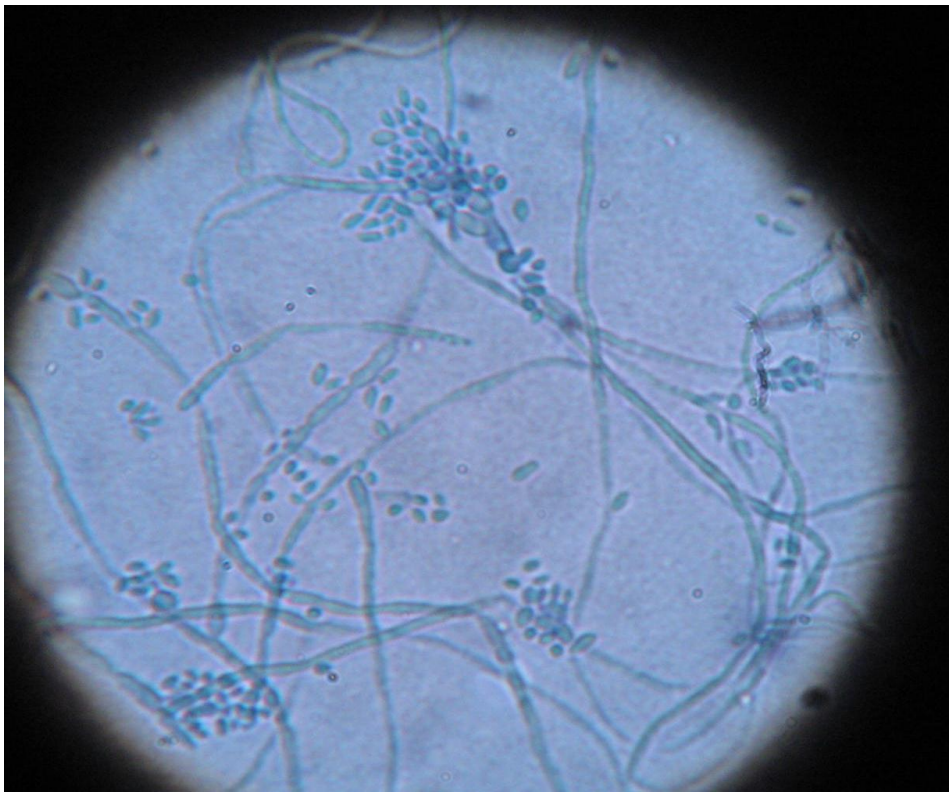
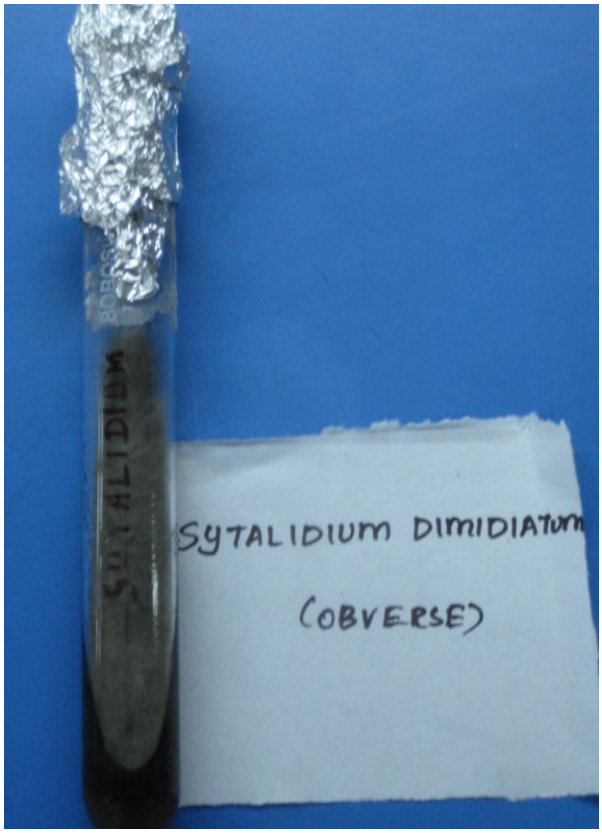
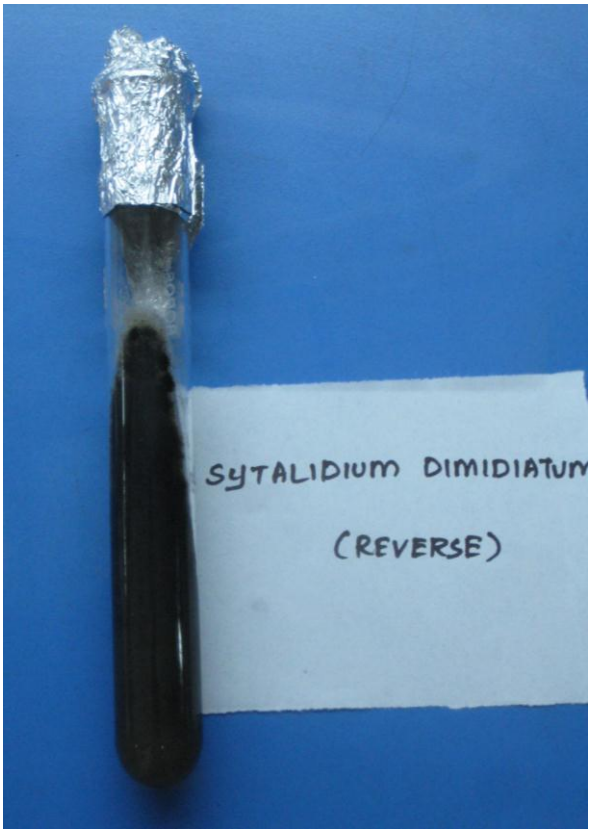


MICROSCOPY

SYTALIDIUM

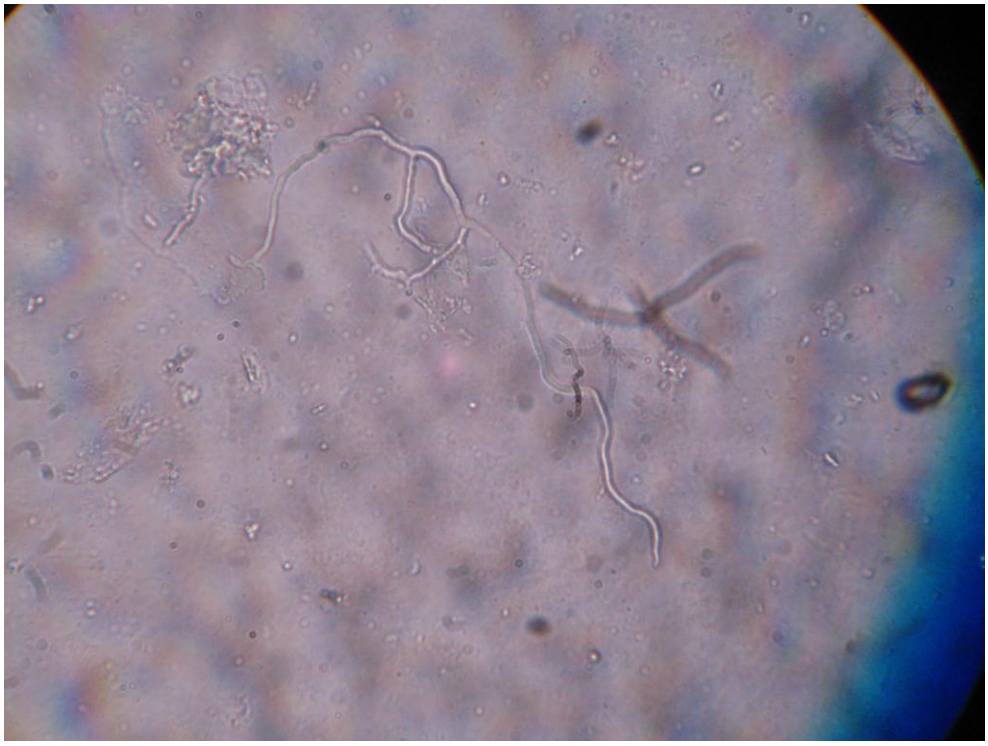




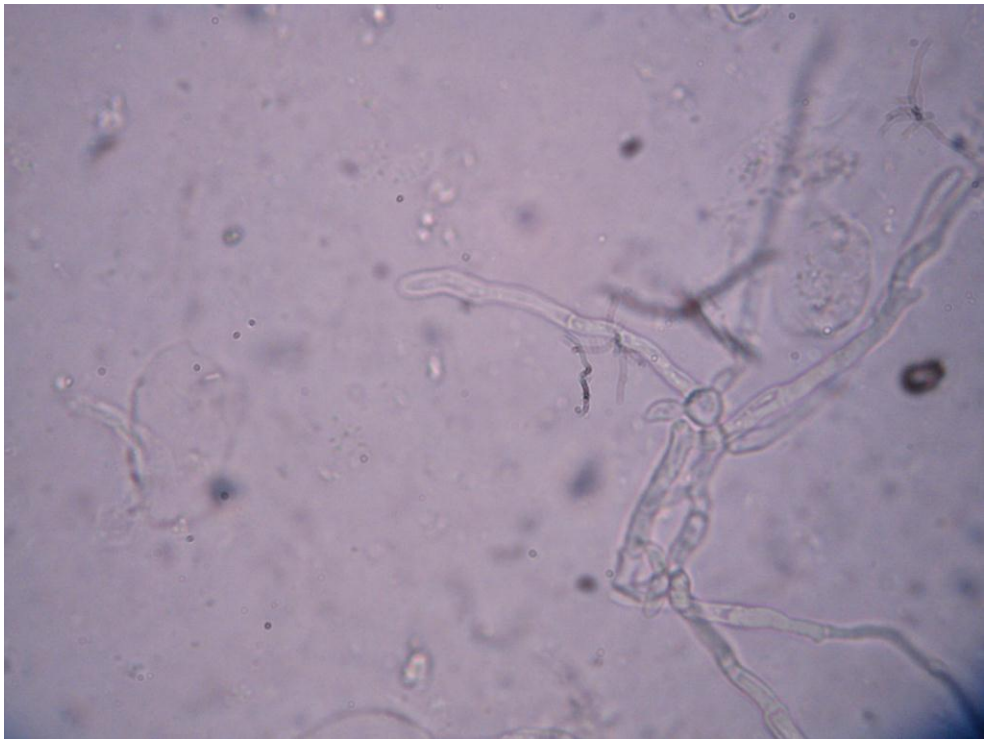
MACROSCOPY

MICROSCOPY

KOH (DMSO)

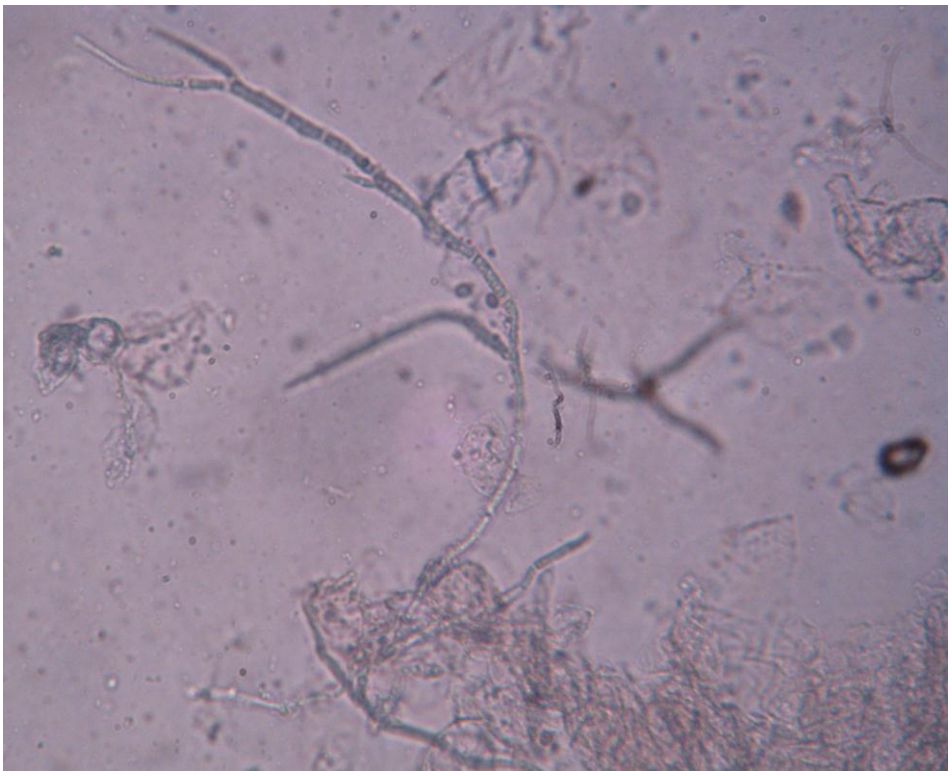


ACUTE BRANCH HYPHAE

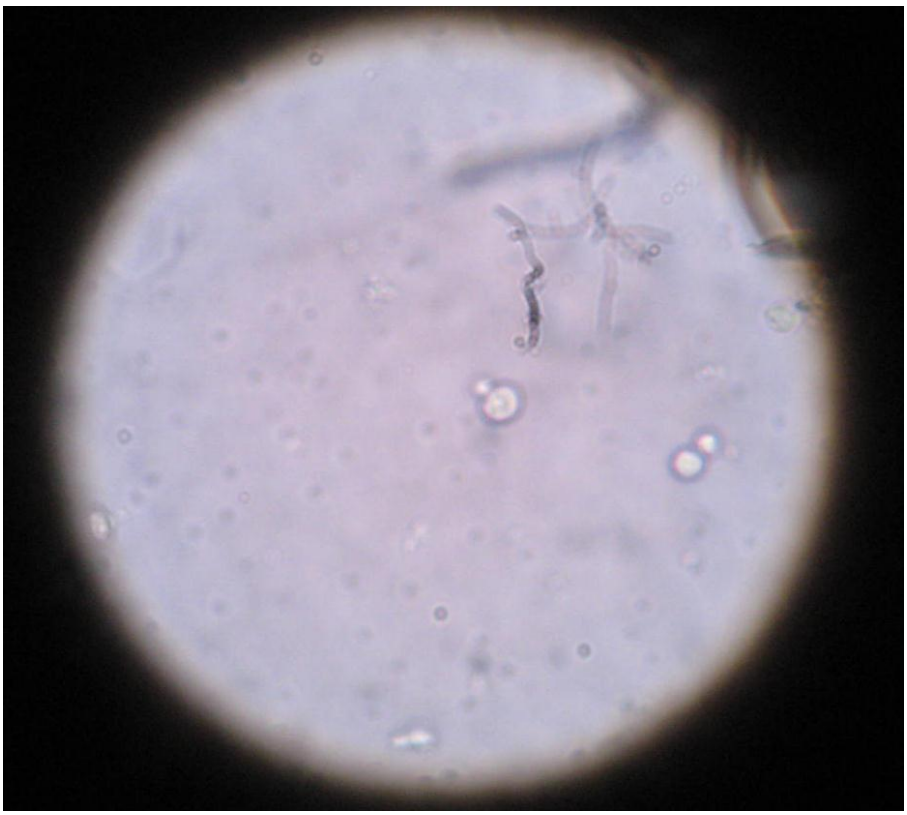


PSEUDOHYPHAE

KOH (DMSO)

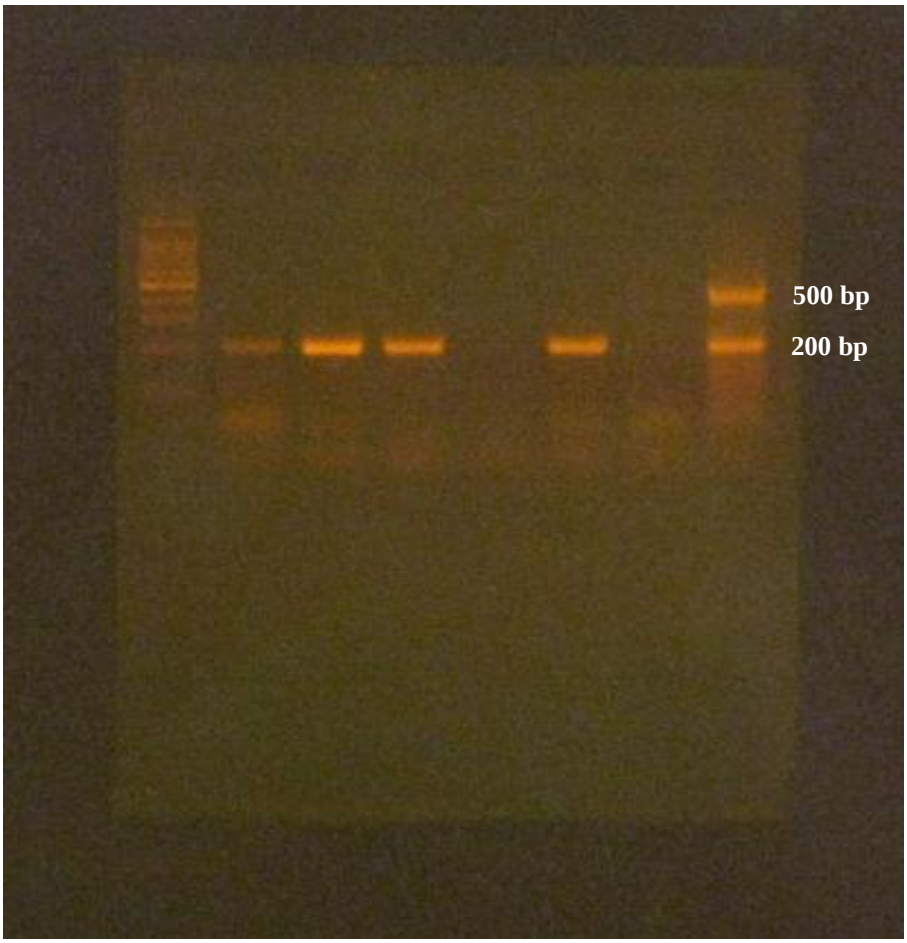


ARTHROCONIDIA



BUDDING YEAST

PCR (ITS 1-TRICHOPHYTON)



Lane 1 - DNA ladder

Lane 2,7 – Trichophyton negative nail sample

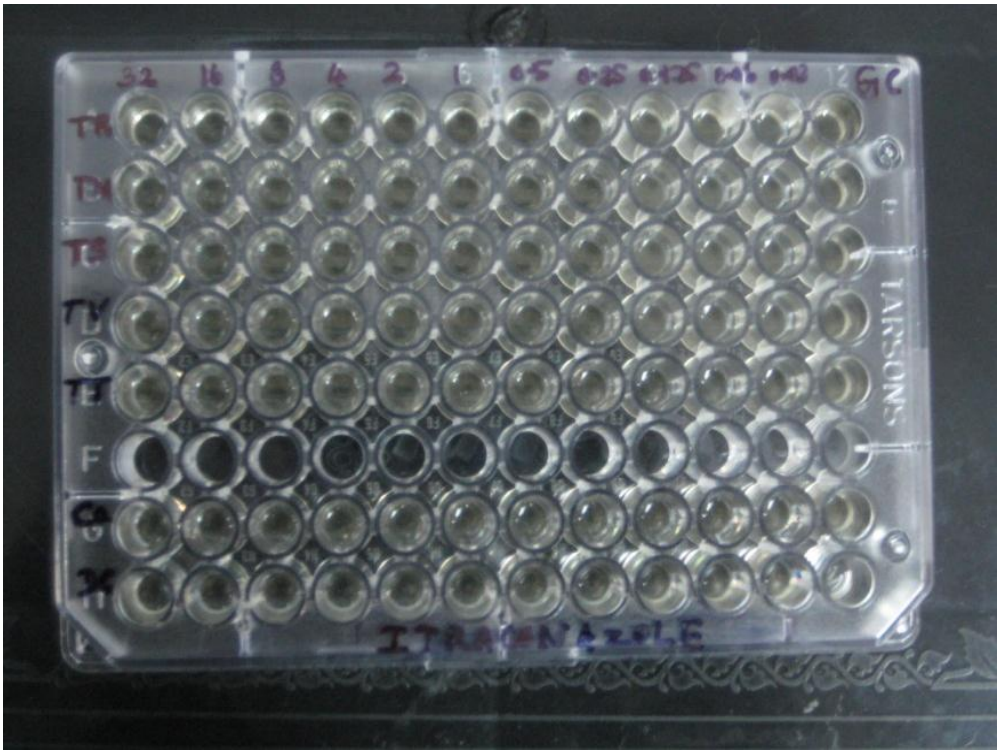
Lane 3 - ATCC T.rubrum 34265(200bp)

Lane 4 - Trichophyton culture(200bp)

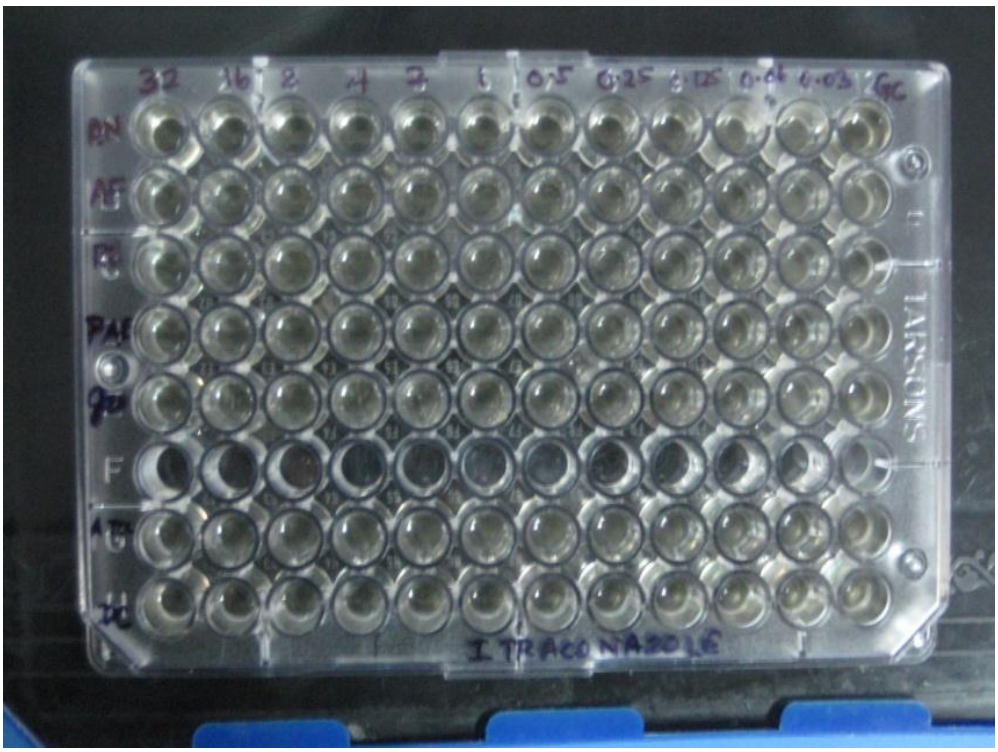
Lane 5 - Non dermatophyte culture

Lane 8 - Trichophyton positive nail sample(200bp, 500bp)

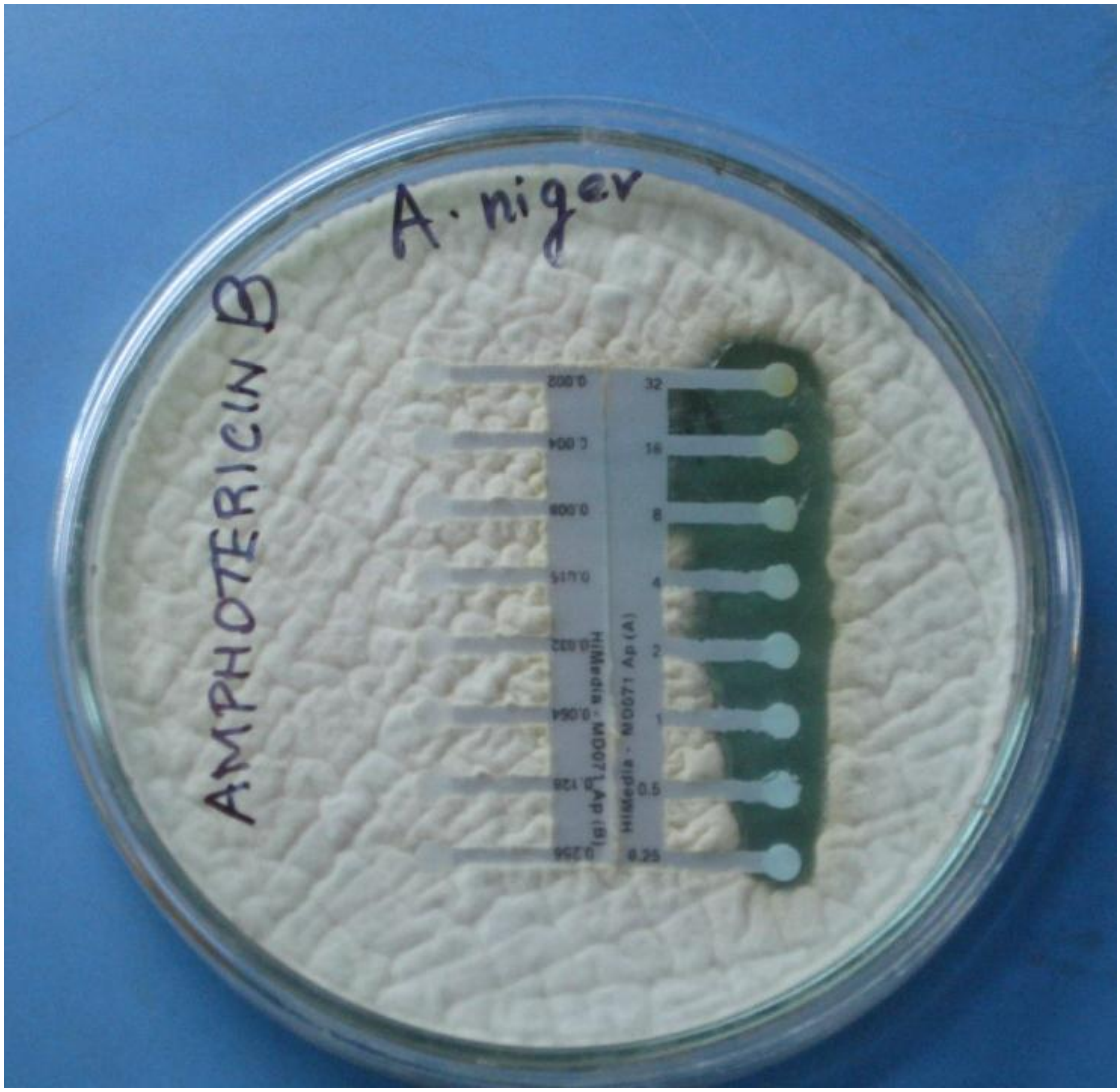
MICROBROTH DILUTION



DERMATOPHYTES

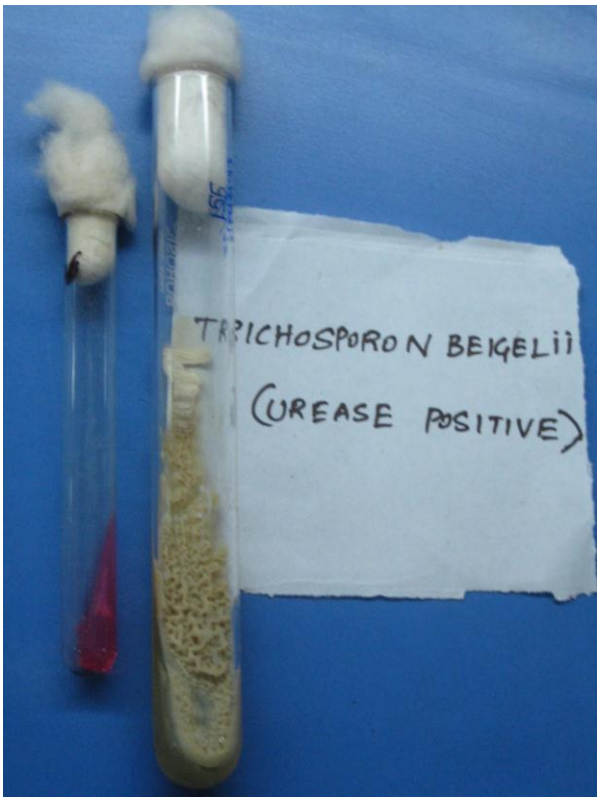


NON DERMATOPHYTES

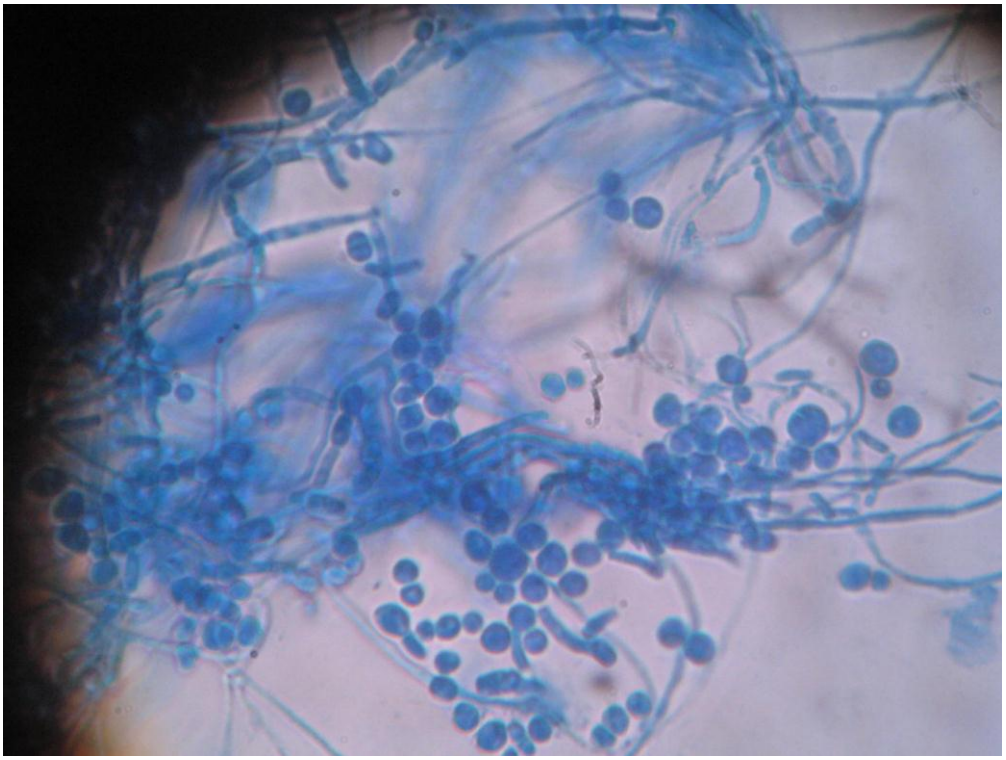


E-TEST

TRICHOSPORON

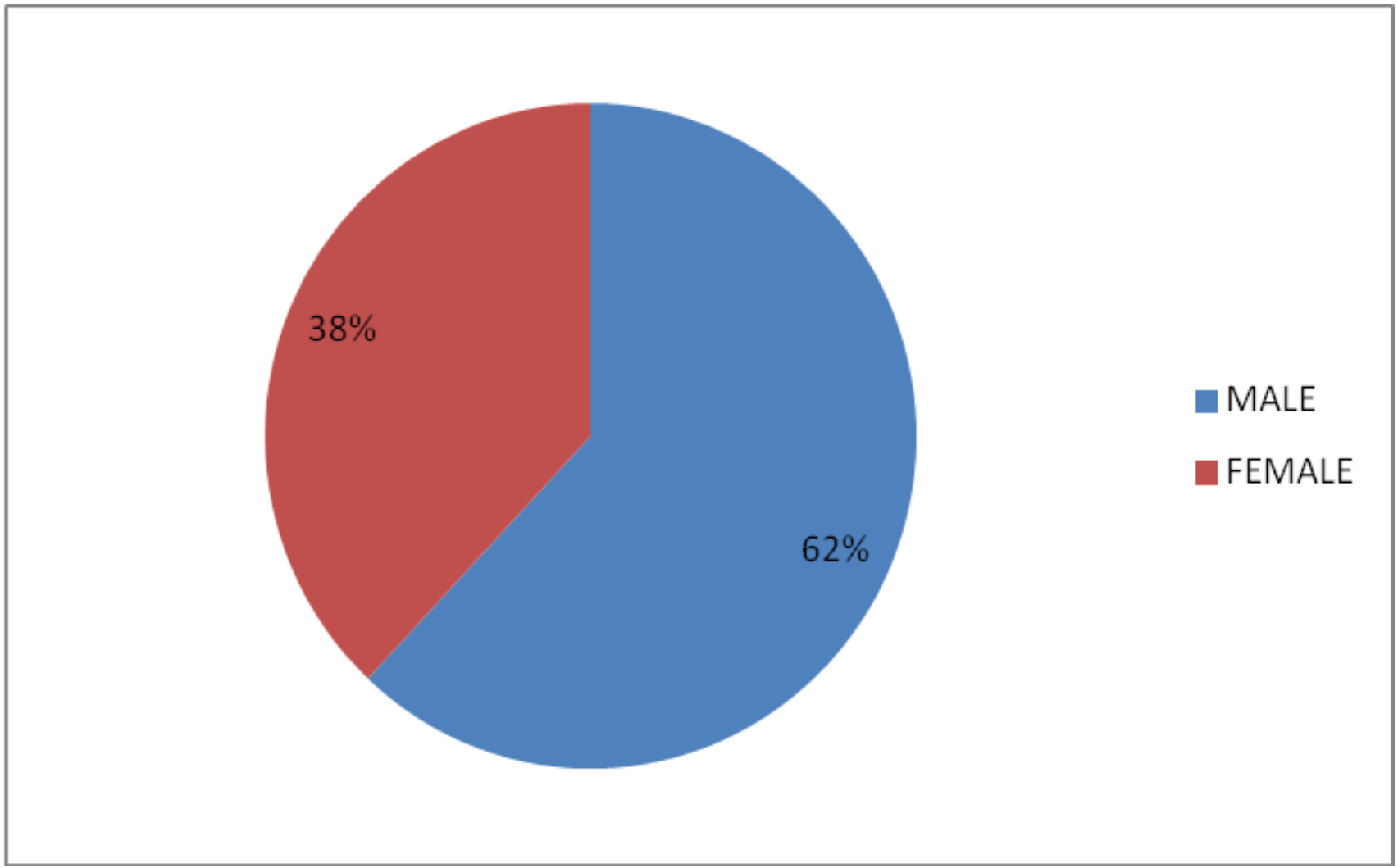


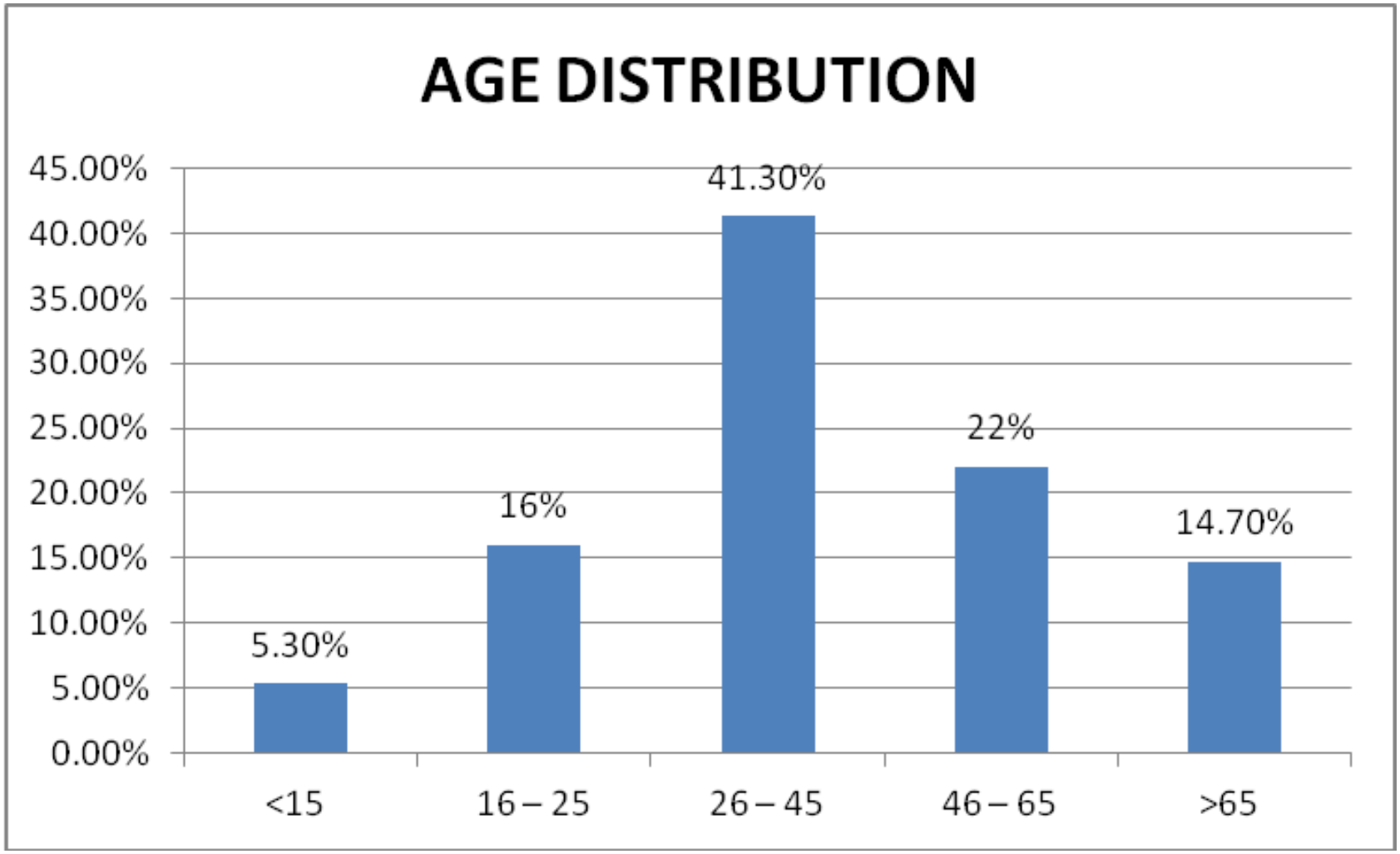
MACROSCOPY

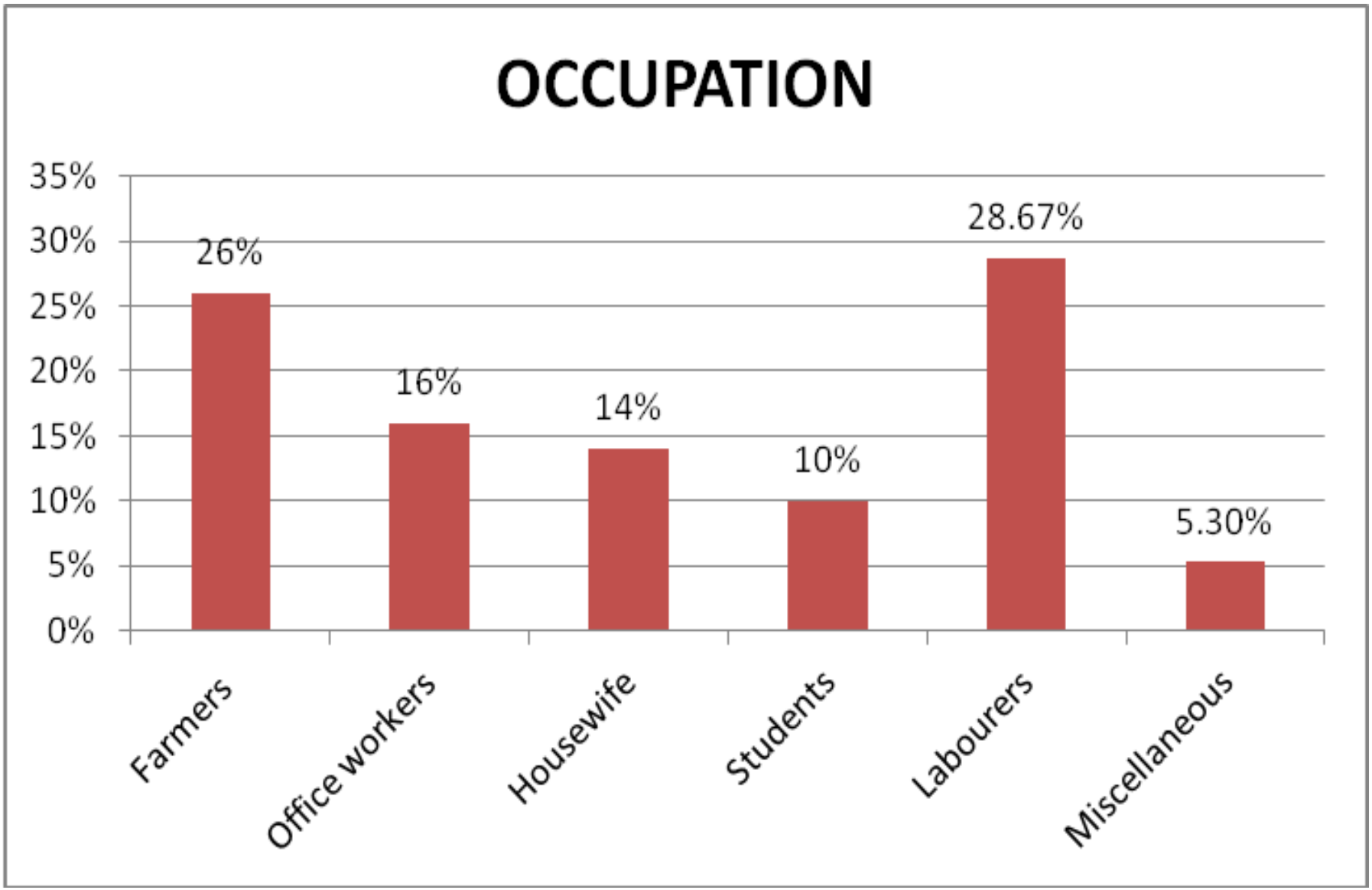


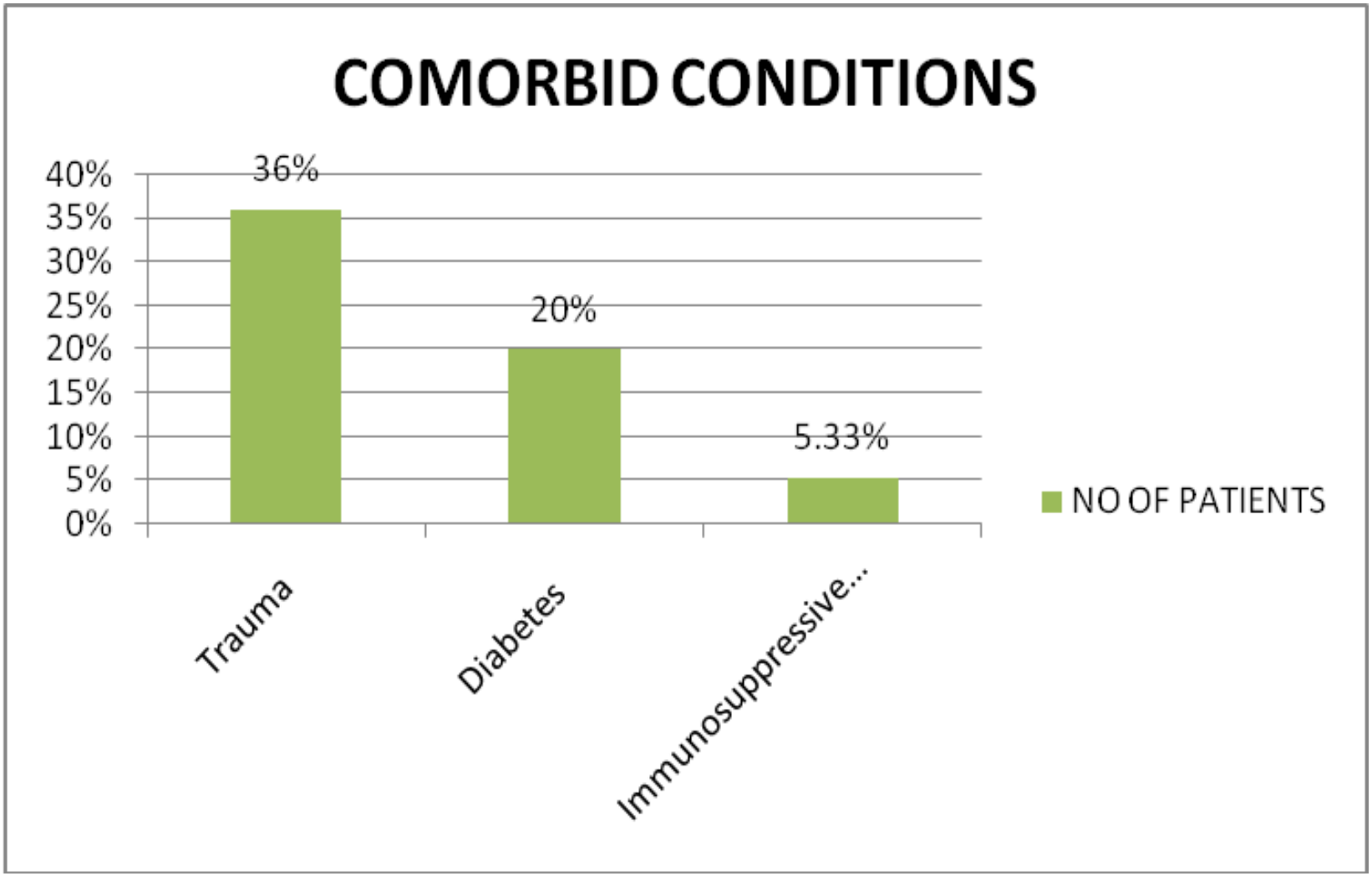
MICROSCOPY

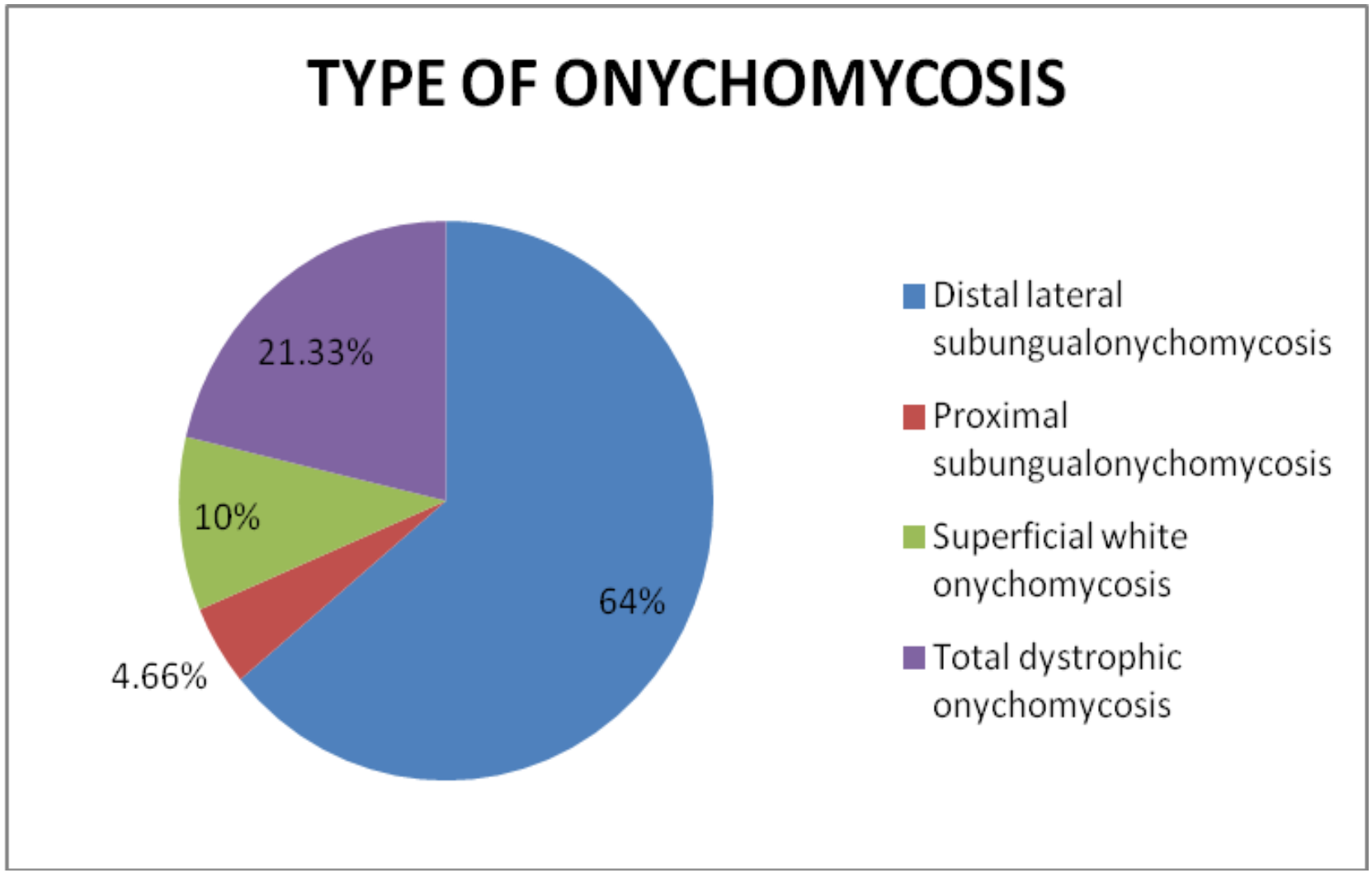
GENDER DISTRIBUTION



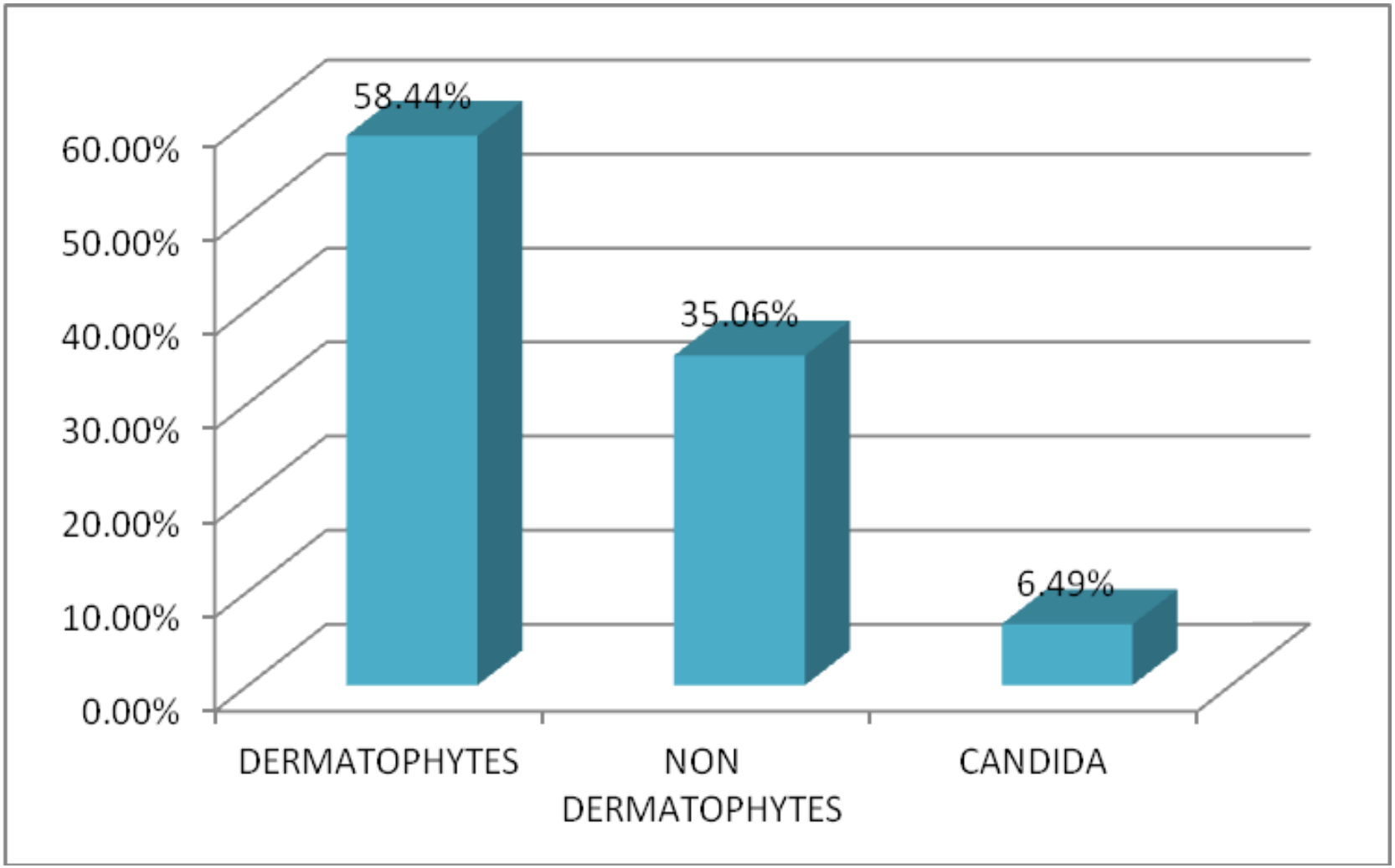

AGE DISTRIBUTION
45.00%
40.00%
35.00%
30.00%
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
5.30%
16%
41.30%
22%
14.70%
<15
16 – 25
26 – 45
46 – 65
>65

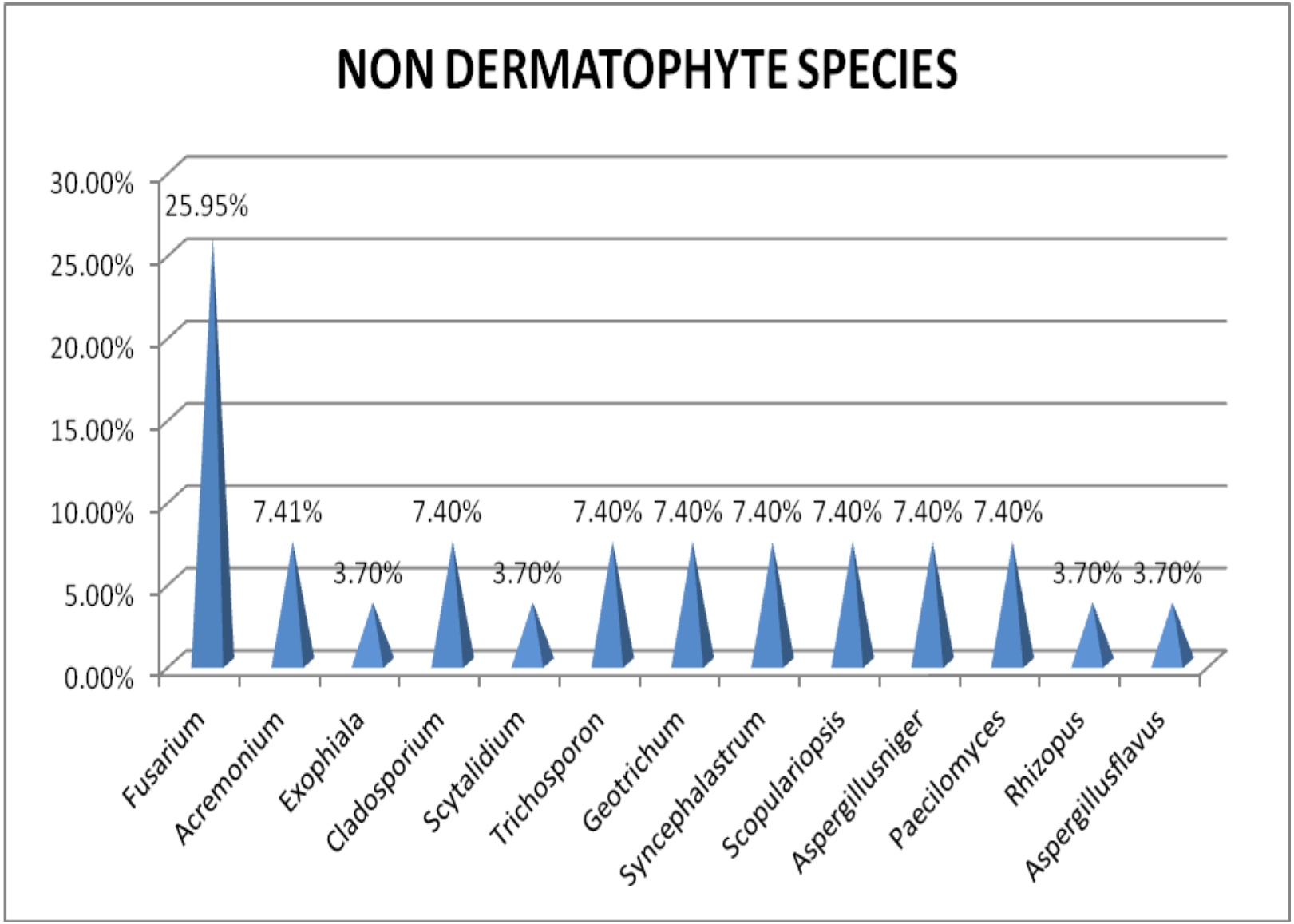

OCCUPATION
35%
30%
25%
20%
15%
10%
5%
0%
26%
16%
14%
10%
28.67%
5.30%
Farmers
Office workers
Housewife
Students
Labourers
Miscellaneous

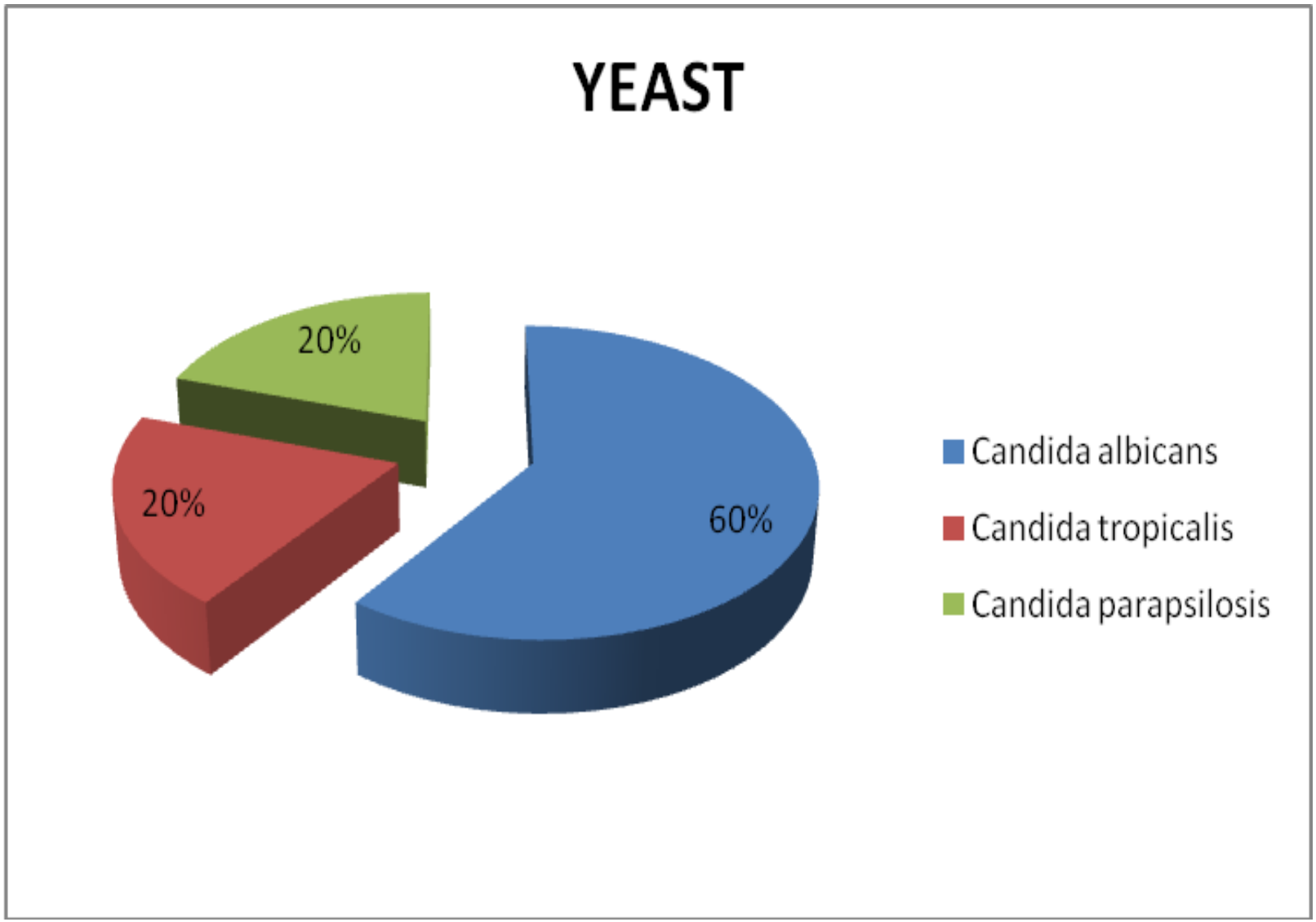

COMORBID CONDITIONS
40%
35%
30%
25%
20%
15%
10%
5%
0%
36%
20%
5.33%
Trauma
Diabetes
Immunosuppressive...
NO OF PATIENTS


TYPE OF ONYCHOMYCOSIS
21.33%
10%
64%
4.66%
Distal lateral subungualonychomycosis
Proximal subungualonychomycosis
Superficial white onychomycosis
Total dystrophic onychomycosis

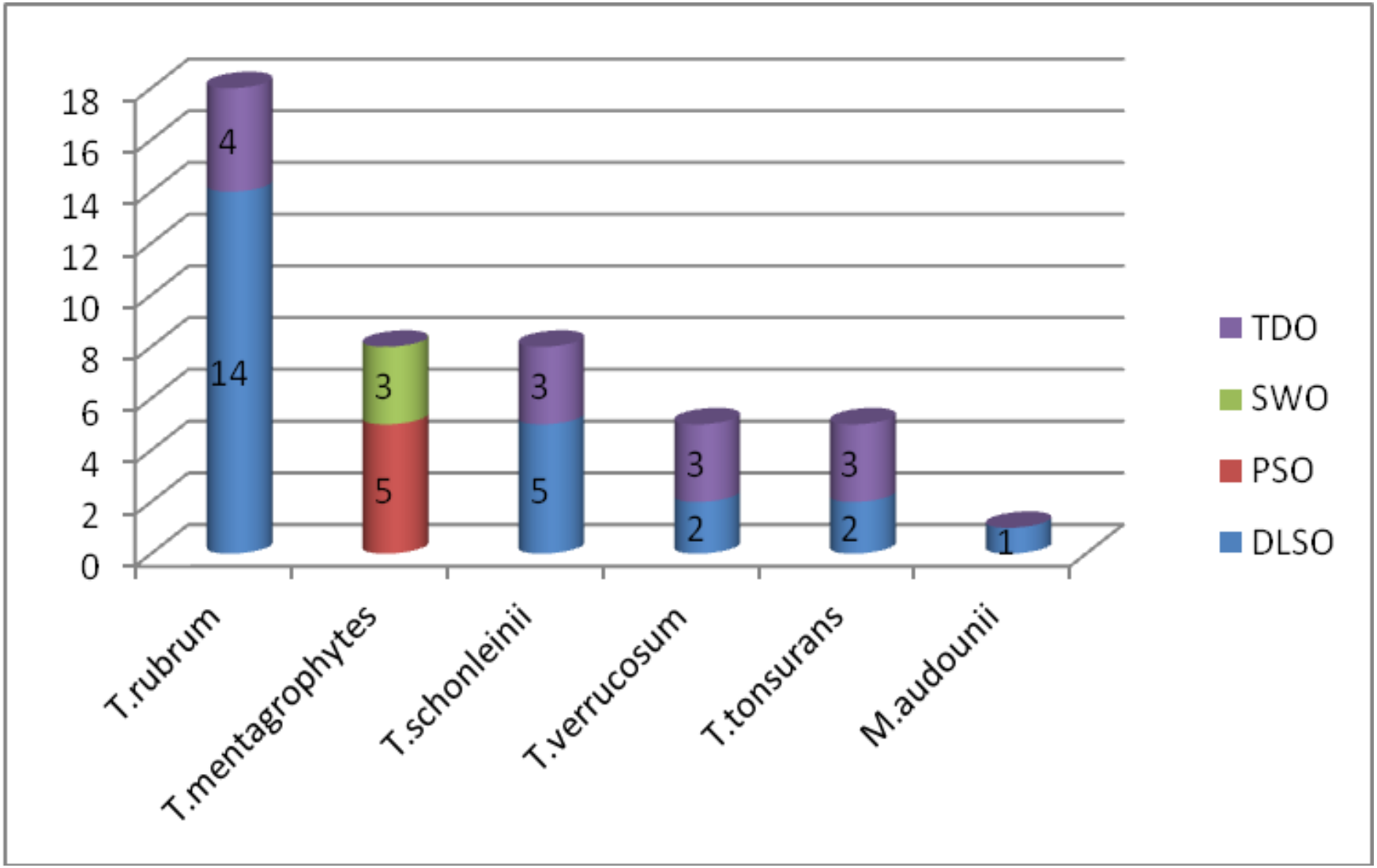
CULTURE ISOLATES



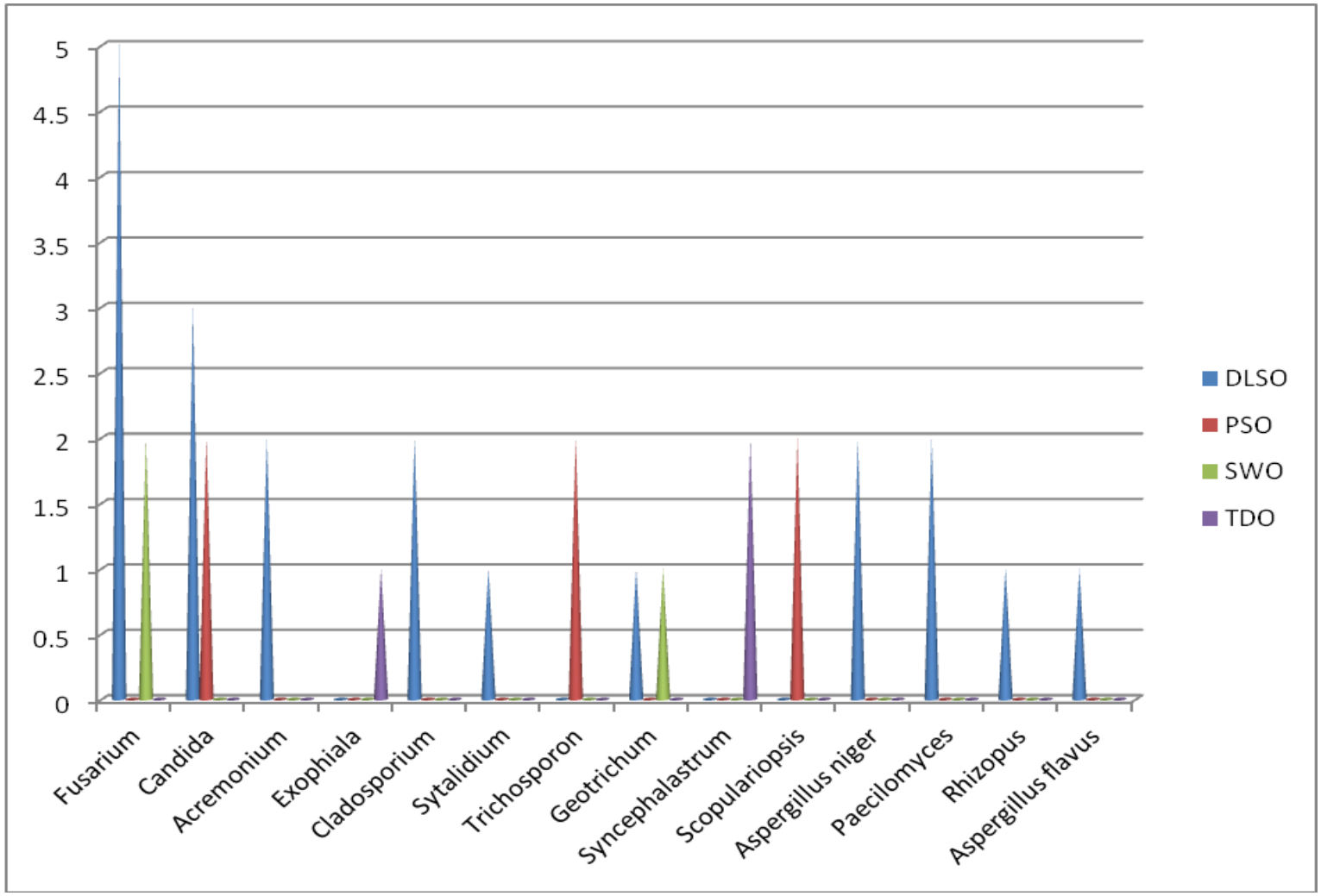

NON DERMATOPHYTE SPECIES
30.00%
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
25.95%
7.41%
3.70%
7.40%
3.70%
7.40%
7.40%
7.40%
7.40%
7.40%
7.40%
3.70%
3.70%
Fusarium
Acremonium
Exophiala
Cladosporium
Scytalidium
Trichosporon
Geotrichum
Syncephalastrum
Scopulariopsis
Aspergillusniger
Paecilomyces
Rhizopus
Aspergillusflavus

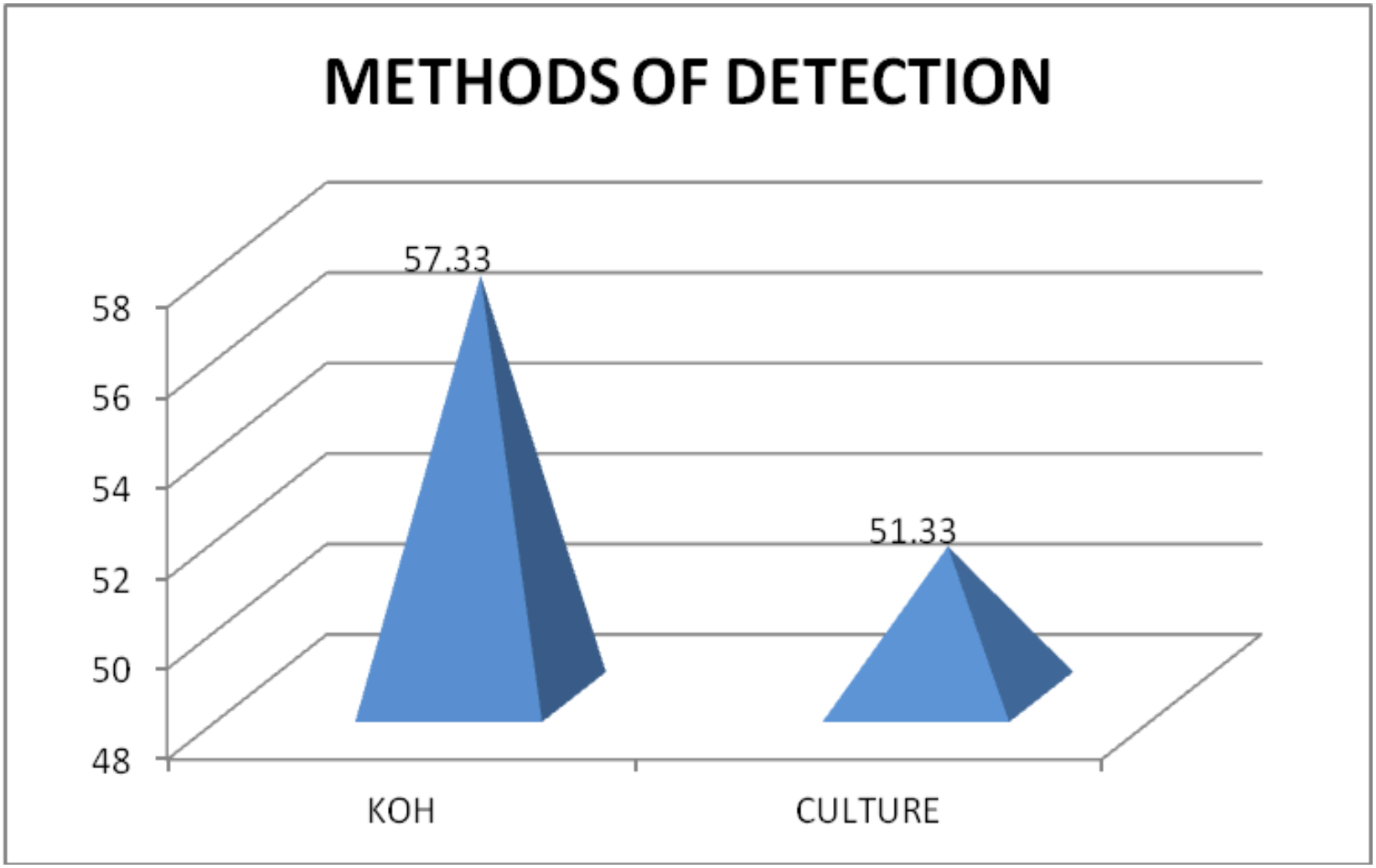

YEAST
20%
20%
60%
Candida albicans
Candida tropicalis
Candida parapsilosis

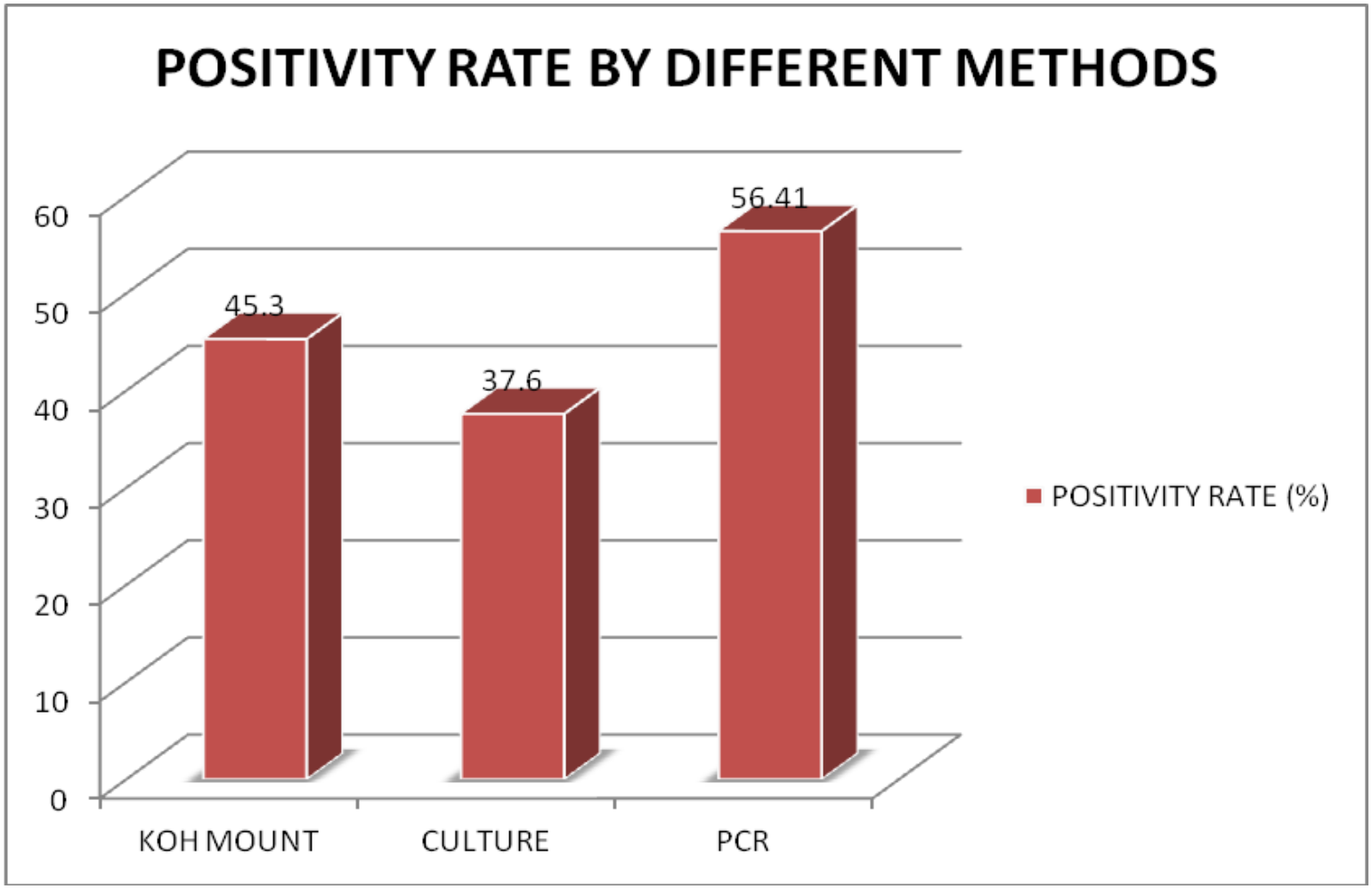
DERMATOPHYTE ISOLATES WITH REFERENCE TO TYPE OF ONYCHOMYCOSIS



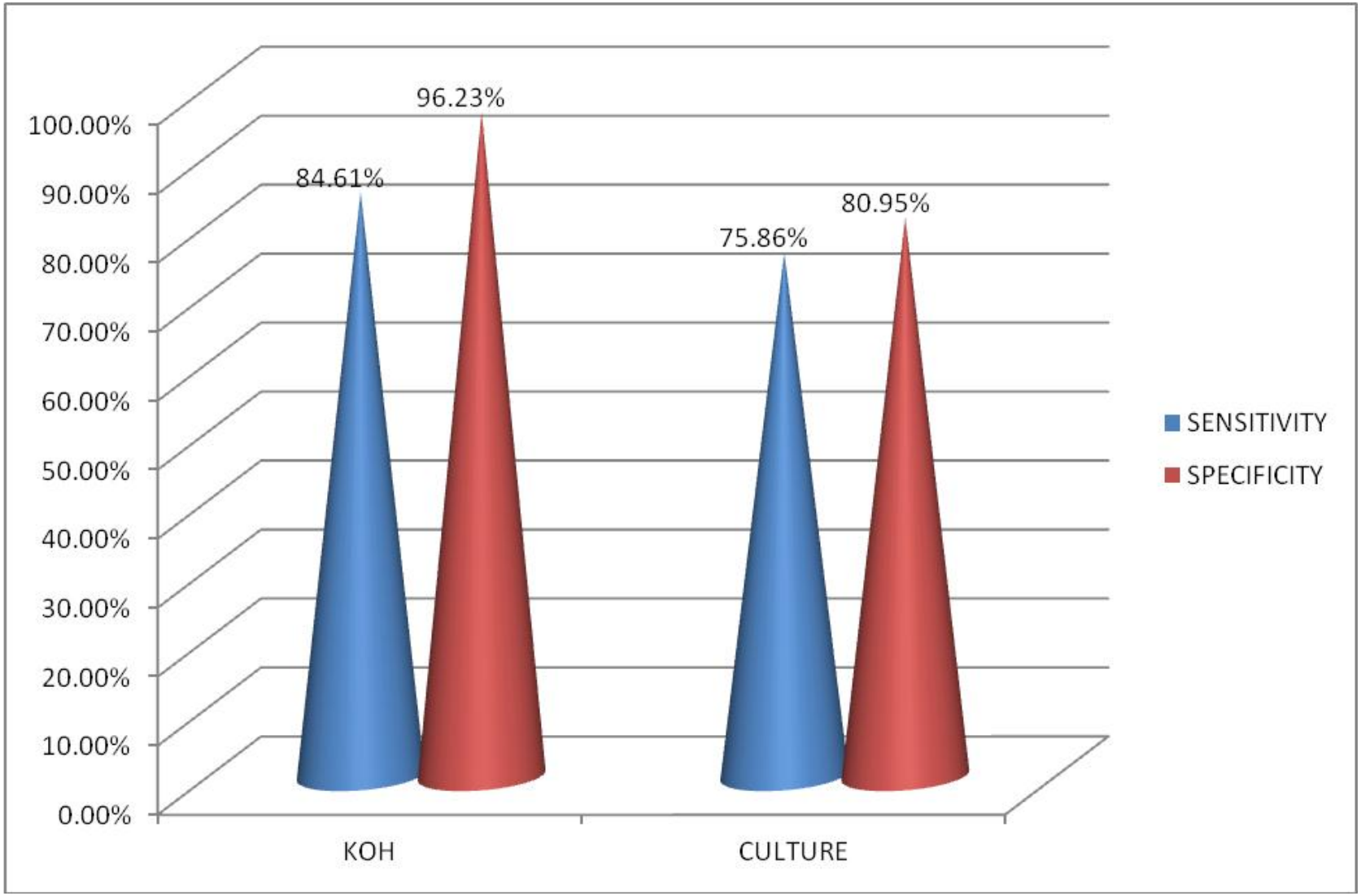
NONDERMATOPHYTE ISOLATES WITH REFERENCE TO TYPE OF ONYCHOMYCOSIS



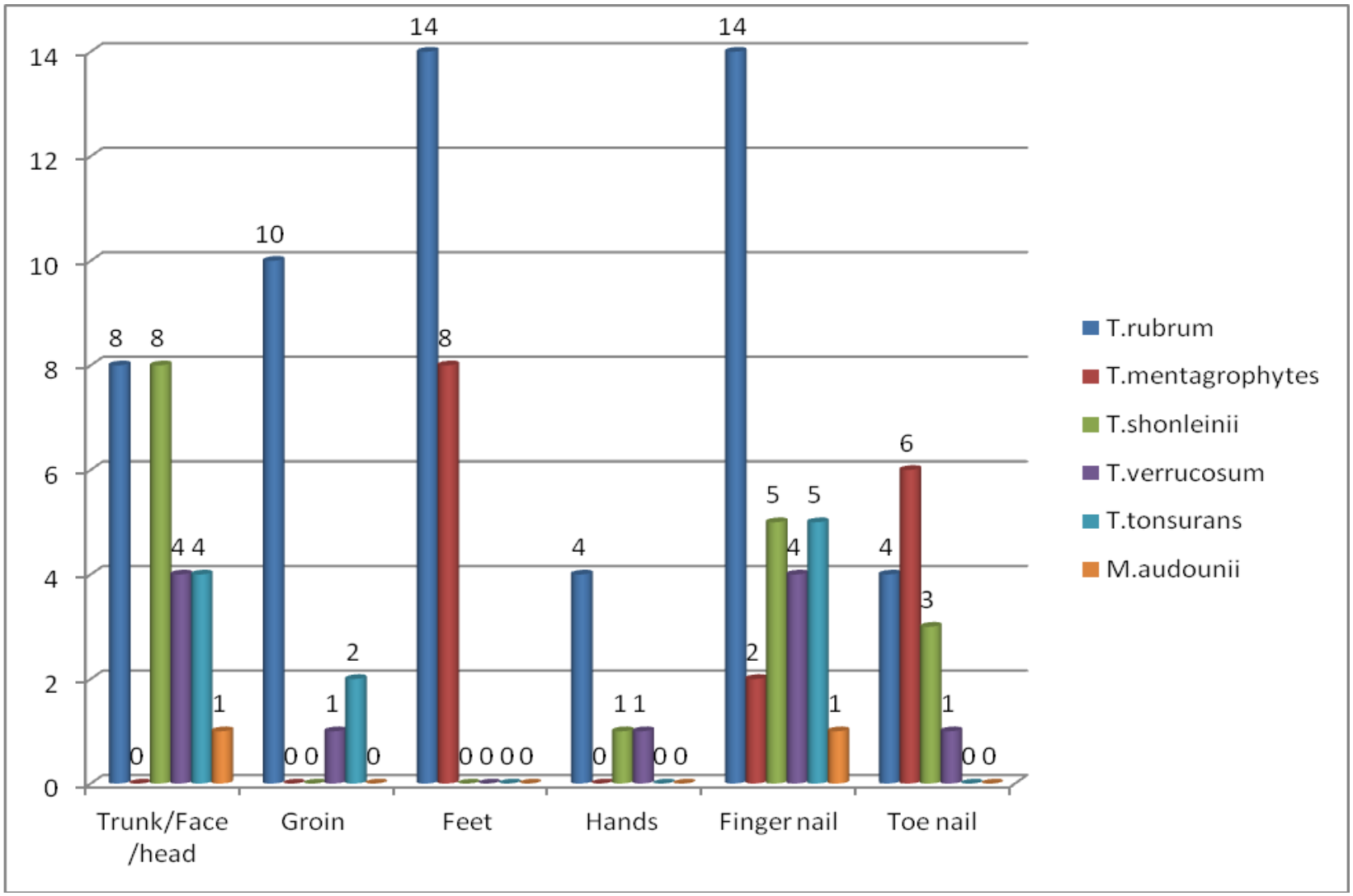

METHODS OF DETECTION
57.33
51.33
58
56
54
52
50
48
KOH
CULTURE


POSITIVITY RATE BY DIFFERENT METHODS
60
50
40
30
20
10
0
45.3
37.6
56.41
KOH MOUNT
CULTURE
PCR
POSITIVITY RATE (%)

ANALYTICAL SENSITIVITY AND SPECIFICITY OF CONVENTIONAL METHODS



DERMATOPHYTE ISOLATES



TITLE : A CLINICOMYCOLOGICAL STUDY OF ONYCHOMYCOSIS, ITS ANTIFUNGAL SUSCEPTIBILITY PATTERN AND RAPID DETECTION OF TRICHOPHYTON GENUS FROM NAIL SAMPLES BY PCR ANALYSIS OF 18S rRNA GENE INTERNAL TRANSCRIBED SPACER REGION

INTRODUCTION

Onychomycosis refers to fungal infection of the nail with the causative agents being dermatophytes, nondermatophytic moulds and yeast. Onychopathies are difficult to treat and need prolonged treatment and therapy is directed by the type of organism.

AIM

This study seeks to improve the knowledge of epidemiology, etiological agents clinicomycological features, its antifungal susceptibility pattern and rapid identification of Trichophyton from nail samples.

MATERIALS AND METHODS

Prospective cohort study

Nail scrappings from one hundred and fifty patients of clinically diagnosed cases of onychomycosis were subjected to KOH with DMSO and culture. PCR was done to detect ITS – 1 region of Trichophyton from nail samples. The antifungal susceptibility pattern was done by microbroth dilution.

RESULTS

DLSO was the predominant type(64%). KOH was positive in 57.33%. Culture positivity is 51.33%. Among the isolates, dermatophytes(58.44%), nondermatophytes(35.06%) and candidal(6.49%) were recovered. The predominant species among dermatophytes was T.rubrum(40%) and among nondermatophytes was Fusarium(25.95%). Candida was isolated in 6.49%. Terbinafine was effective for dermatophytes and voriconazole, amphotericin B for nondermatophytes. Regarding detection of Trichophyton from nail samples, positivity rate of KOH(45.3%), culture(37.6%) and PCR(56.4%). PCR has higher detection rate than KOH and culture. E test shows good agreement with microbroth dilution.

CONCLUSION

Nail changes are not always a reliable marker for predicting causative organisms since only 51.33% showed positivity by culture. Nondermatophytes and yeast which were previously regarded as contaminants are now emerging as pathogens. Newer diagnostic methods are the need of the hour not only for accurate diagnosis but also for post therapeutic strategies.